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The
Scientific Association
Dedicated
to
Analytical Excellence

AOAC
INTERNATIONAL

JAINEE 75(3)375–590 (1992)
ISSN 1060-3271

TEN SECOND CLEAN UP COLUMN

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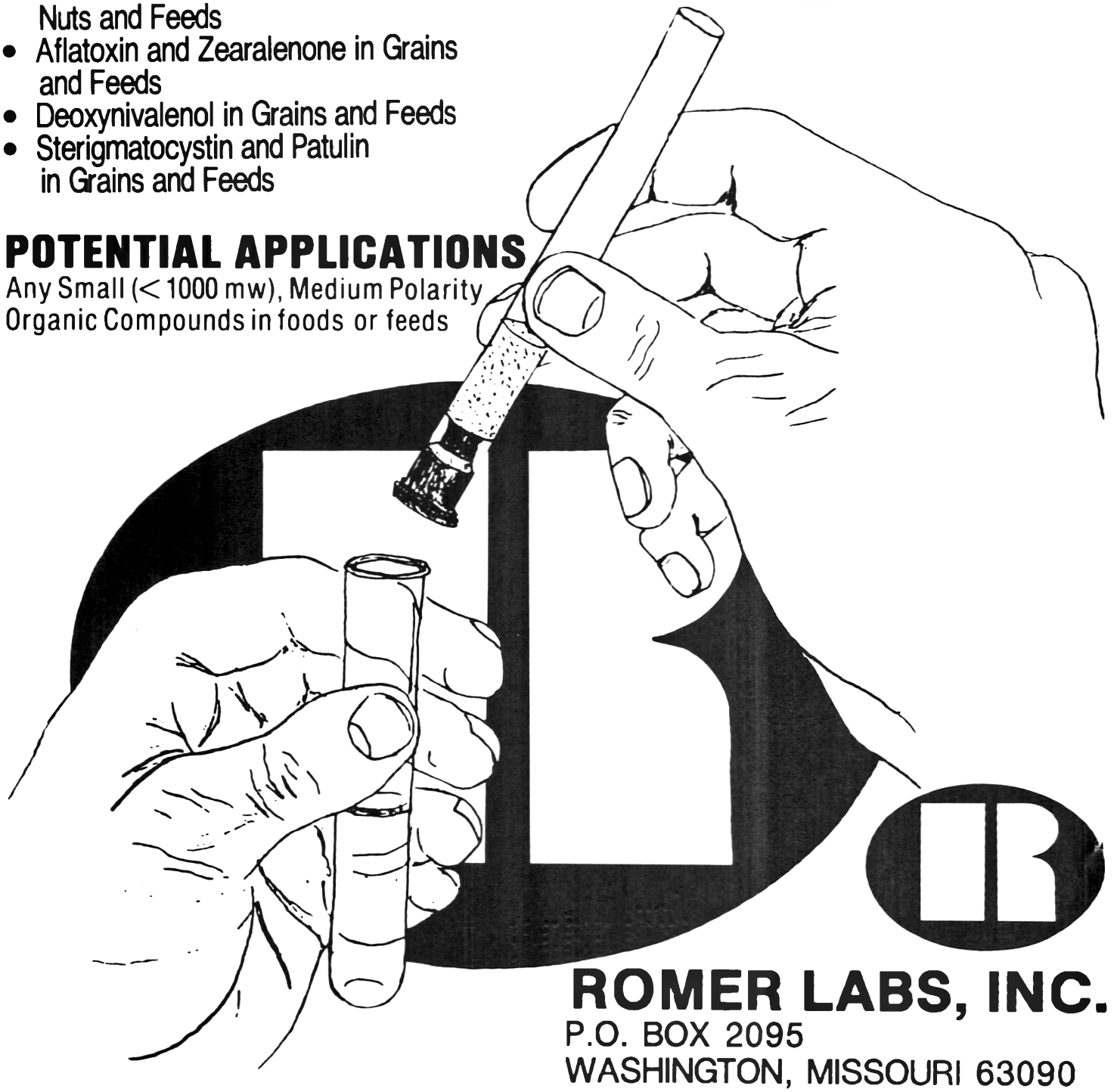

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JOURNAL OF AOAC INTERNATIONAL

A Peer-Reviewed Journal Publishing Basic and Applied Research in the Analytical Sciences Related to Foods, Drugs, Agriculture, and the Environment

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For Your Information

Meetings

May 28–30, 1992: AOAC Official Methods Board Meeting, St. John's, Newfoundland, Canada. Contact: Nancy Palmer, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

June 2–4, 1992: AOAC Board of Directors Meeting, AOAC, Arlington, VA. Contact: Nora Petty, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

June 8–10, 1992: Midwest Regional Section Meeting, Champaign, IL. Contact: Karen Harlin, University of Illinois, Dept of Veterinary Bioscience, 2001 S. Lincoln, Urbana, IL 61801, telephone 217/244-1569.

June 17, 1992: MidCanada Regional Section Meeting/AOAC Day, Winnipeg, Manitoba, Canada. Contact: Tom Nowicki, Canadian Grain Commission, 1401–303 Main St, Winnipeg, MB, R3C 3G1, Canada, telephone 204/983-3345.

June 24–26, 1992: Pacific Northwest Regional Section Meeting, Olympia, WA. Contact: Norma J. Corristan, Oregon Dept of Agriculture, 635 Capitol St NE, Salem, OR 97310-0110, telephone 503/378-3793.

August 30–September 3, 1992: 106th AOAC Annual International Meeting and Exposition. Cincinnati, OH. Contact: AOAC Meetings Dept, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

August 30 and September 4, 1992: AOAC Board of Directors Meeting, Cincinnati, OH. Contact: Nora Petty, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

November 17–20, 1992: Central Regional Section Meeting, Kalamzaoo, MI. Contact: Sungsoo Lee, Kellogg Co., 235 Porter St, PO Box 3423, Battle Creek, MI 49016-3423, telephone 606/961-2823.

February 1–4, 1993: Southeast USA Regional Section Meeting, Atlanta, GA. Contact: Jan Hobson, Griffith Corp., Rocky Ford Rd, PO Box 1847, Valdosta, GA 31603-8635, telephone 912/242-8635.

March 29–30, 1993: Europe Regional Section Meeting, Barcelona, Spain. Contact: J. Sabater, Laboratorio Dr. J. Sabater Tobella, Calle de Londres 6, 08029 Barcelona, Spain, telephone 34/3-410-9343

July 25–29, 1993: 107th AOAC Annual International Meeting and Exposition, Washington, DC. Contact: AOAC Meetings Dept, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

Report on AOAC Europe Section Second International Symposium—Protection of Public Health: A Challenge for Food and Environmental Analysts

The theme of the second international symposium organized by the AOAC Europe Section held in Maastricht, The Netherlands, November 12–13, 1991, was the protection of public health through food and environmental analysis.

In his keynote presentation, Dr. Schuring of The Netherlands outlined the current food law enforcement situation within EEA (EC plus EFTA (European Free Trade Association)). The existing systems are both diverse and complex. In some countries, as many as 3 government departments are involved in the administrative control while the laboratory control may be at central, regional, or local levels or at various combinations of these 3 levels.

Food and beverages play an important role in trade and in the Single Market; they account for 30% of all goods traded. EC took the first steps toward harmonization of food laws in 1989 with the adoption of Council directive 89/397/EEC "on the official control of foodstuffs." This directive lays down the framework for food law harmonization within EC and outlines the means by which this objective will be achieved. Very recently, people engaged in Food Law Enforcement within EEA formed an informal organization called Food Law Enforcement Practitioners (FLEP). The aims and objectives of FLEP include *(1)* preparation of a register of people engaged in food law enforcement listing their respective areas of expertise; *(2)* laboratory quality assurance; *(3)* exchange of technical/non-technical information, analytical expertise, etc.; *(4)* and investigation of the possible implementation of the HACCP system for food control.

The second keynote paper presented by Dr. van der Berg of The Netherlands concentrated on environmental policies for health protection within Europe. The speaker gave an overview of the complex legislative scene relating to chemical substances. He indicated that the initial thrust was market harmonization, but risk assessment is now playing an increasingly important role. Many other environmental directives exist, including those concerned with the quality of air and water.

Trace Element Analysis.—Advances in technology and the introduction of hyphenated techniques have afforded the analyst with the opportunity to separate, identify, and quantify the actual form(s) in which trace elements exist in the sample matrix. Professor Astruc of Pau, France, concentrated on sample preparation aspects of speciation analysis and referred to work undertaken on Hg and Sn in the aquatic environment. The solubility and extractability of the various species that each element can form are extremely variable parameters and present many sample preparation

difficulties. For water, sample preparation is not normally problematic. Sediments and fish, however, may require a variety of extraction procedures and pre-chromatographic clean-up steps.

Professor Ebdon of Plymouth, UK, gave an account of coupling techniques in use and an illustration of their practical application in the workplace. GC-AAS is suitable for volatile species, such as Hg and Se, while the non-volatiles, such as As and Sn, require the use of HPLC. Problems which arise from coupling the above systems were discussed and solutions offered. Levels of 20 parts per trillion of tributyltin (TBT) in water can be achieved by using HPLC-FAAS with the addition of a simple interface and a slotted tub-in flame atom trap. Lower levels of TBT require ICP-MS; an optimum coupling for this technique was described.

Flue gases and work place atmospheres were considered by Professor Klockow of Dortmund, Germany. Air and flue gases are complex mixtures of gaseous and particulate compounds (normally referred to as aerosols) in which the droplets and solid particles are suspended in a carrier gas. Prior to metal speciation analysis of aerosols, a proper sampling plan is required. This plan must ensure that reliable separation and pre-concentration of the gaseous and particulate metal compounds can be achieved without artifact formation.

Organic Contaminants.—Dr. Pfannhauser of Vienna, Austria, concentrated on polycyclic aromatic hydrocarbons in food. PAHs are mainly anthropogenic in origin. They are the products of incomplete combustion and arise from various sources, such as motor car exhaust, industrial incineration, and the burning of household organic waste matter. The amount of PAHs which man can ingest is controlled by a variety of factors, such as location of raw material production (open farmland or industrial area), season, diet, and climate. In 1990, the Austrian Federal Ministry of Health sponsored a research project to ascertain the contribution made by vegetables to the PAH content of the total diet.

The second paper in this session was presented by Dr. van Zoonen of RIVM, The Netherlands, who concentrated on the determination of pesticides in food using coupled chromatographic techniques. These techniques are amenable to automation and have the potential to enhance both sensitivity and selectivity. The application of LC-LC to varying matrixes was presented. ETU and related pesticides in water were cleaned up on one column by using the "heart cutting" technique and separated on the second column using a different mobile phase. The extension of this technique to apple juice necessitated the introduction of a precolumn SPE step. The development of a multiresidue system for 9 permissible pesticides in cereals was also described.

Traceability of measurement was discussed by Mr. Wagstaff of BCR, EEC. He outlined the projects in the food and environmental areas that are either completed or underway, and the reference materials that have been certified.

Biotechniques in Food and Environmental Analysis.—Food safety plays a vital role in public health, and each year a number of people die from food-borne diseases and many more are hospitalized. Classical microbiological methods are labor intensive and time consuming, and frequently the food has been consumed before the analytical data becomes available. Professor Huis of TNO, The Netherlands, dealt with the changing scene in this area where both automation and biotechnology are leading to an increase in throughput and rapid turnaround time for analytical data. Techniques/instrumental methods discussed included those based on conductance, impedance, bioluminescence, immunoassays, and DNA. Using some of the above, it is now possible to moni-

tor and measure the sources of microbiological contamination of food during the various production stages. In the production of poultry produce, continuous monitoring of the feed, housing, hygiene, slaughterhouse, and final product are now possible and as soon as contamination is detected, corrective action can be taken.

Biosensors, which are composed of a biological selector coupled directly to a detector, offer a simple and selective measurement technique.

TNO and the University of Nijmegan have developed a bio-electro catalytic sensor. In this instance, a redox enzyme is coupled to an electron conducting polymer, which, in turn, is linked to an amperometric detector. A practical application of this involves using the redox enzyme-glucose oxidase for the continuous monitoring of glucose in the fermentation industry. These sensors can be used for several months without loss of sensitivity; they have a fast response time and are independent of the concentration. The stability of the enzyme is also improved by the fact that it can be turned "on and off."

AOAC—AFDO Cooperative Program Initiated with QA Short Course

As the first project in a program to actively cooperate in joint ventures, AOAC International and the Association of Food and Drug Officials (AFDO) are jointly offering the 2-day AOAC Quality Assurance for Analytical Laboratories Short Course on June 17 and 18, 1992, at the AFDO Annual Meeting in Buffalo, NY.

For this special scheduling of the course, a $295 registration rate is being offered to both AFDO and AOAC members (the regular AOAC member price is $495). Non-members will be charged the current AOAC non-member rate of $560.00.

For registration forms or further information, contact AFDO, telephone 717/757-2888, fax 717/755-8089, or the AOAC Administration and Meetings Dept, telephone 703/522-3032, fax 703/522-5468.

AOAC Research Institute Board of Directors Meeting

The newly elected Board of Directors of the AOAC Research Institute met for the first time February 24, 1992, at AOAC headquarters. Members of the Board were elected by the Board of Directors of AOAC International, which is the sole member of the Research Institute. Board members are Ronald A. Case, Anthony M. Guarino, P. Frank Ross, Lawrence A. Roth, and H. Michael Wehr. As its first order of business, the Board elected Wehr and Ross as chair and vice-chair for the term ending with the annual meeting. Ronald R. Christensen, as AOAC International Executive Director, serves as permanent secretary-treasurer of the Board.

Actions taken by the Board included ratification of the Certificate of Incorporation and the Bylaws for the AOAC Research Institute; amendment of the bylaws to increase the number of directors from 5 to 7 to allow more diverse representation; and authorization to execute several agreements with AOAC International for facilities and services and license to use the AOAC name.

The Board also reviewed in depth the documentation and budget for the Test Kit Performance Testing Program and made several suggestions and requests for operational policy and budget items.

Program Initiation.—See "Update: AOAC Research Institute" in the February, 1992, issue of *The Referee* for details of the first phase of the Test Kit Performance Testing Program. Applications will be accepted first for review of kits intended to determine β-lactams, tetracylines, sulfonamides, and gentamicin residues in milk. Applications may be submitted beginning April 1, 1992. This date has been revised from the date previously announced because of the need to resolve uncertainties in the regulatory requirements for the designated kits.

This first phase will be done in cooperation with the Center for Veterinary Medicine of the U.S. Food and Drug Administration and will focus on kits intended to be used in regulatory milk screening programs. Applicants are responsible for ensuring that the specifications and claimed performance for a submitted kit meet the regulatory requirements for the kit's intended use, and that the testing protocol developed by the AOAC Research Institute is an appropriate test for those specifications. Applicants in other national market areas must verify compliance with the particular national specifications the kit is intended to meet.

As a result, previously announced data requirements have been expanded to include performance data on incurred residues, and differentiation of positive and negative samples at safe levels.

Although drug residue screening is done on fresh milk, submission of data on previously frozen milk is encouraged. Because of the need to maintain stable test samples for the program, kit performance on incurred residues will be verified using previously frozen milk.

Application packages or further information may be obtained from the AOAC Research Institute, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301 USA, telephone 703/522-2529, fax 703/522-5468.

Books in Brief

Good Laboratory Practice Standards: Applications for Field and Laboratory Studies. Edited by Willa Y. Garner, Maureen S. Barge, and James P. Ussary. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1992. 571 pp. Price: U.S. & Export: $89.95. ISBN 0-8412-2192-8.

What should you be doing to meet the EPA's Good Laboratory Practice (GLP) Standards regulations? This new volume tells you with concrete ideas for establishing a compliance program and for refining the compliance process. Its 33 chapters, written by experienced quality assurance professionals, outline approaches that have resulted in successful compliance. The authors, representing industry, field and laboratory research, and government agencies, also describe methods for avoiding some common mistakes. Included are sections on GLP requirements, quality assurance responsibilities, computer validation, special studies, and regulatory impact. Of special interest are appendices containing the text of the GLP Standards and the recently established EPA penalties for non compliance.

Handbook of Inductively Coupled Plasma Mass Spectrometry. Edited by K.E. Jarvis, A.L. Gray, and R.S. Houk. Published by Blackie & Sons, Ltd, Academic & Professional Division, Bishopbriggs, Glasgow G64 2NZ, UK, 1991. 392 pp. Price: £75.00. ISBN 0-2169-2912-1.

Inductively coupled plasma mass spectrometry (ICP-MS) is a new analytical technique that has gained rapid and wide acceptance in many fields. Experience of ICP-MS in many laboratories is limited; therefore, there is a need for a handbook covering not only the theory of operation, fundamentals, and history of the technique, but including practical information and tips that allow the reader to make best use of the instrumentation available. Comprehensive coverage is given of critical areas such as sample preparation, sample introduction, solids analysis, and isotope ratio measurements.

Food Safety Assessment. Edited by John W. Finley, Susan F. Robinson, and David Jon Armstrong. Published by the American Chemical Society, 1155 16th

St, NW, Washington, DC 20036, 1992. 478 pp. Price: U.S. & Export $99.95. ISBN 0-8412-2198-7.

Providing the latest information from academia, industry, and government, this volume offers a comprehensive examination of the principles and issues involved in the safety evaluation of foods and ingredients, as well as of new processes for the manufacturer and distribution of food products. Its 35 chapters are divided into 8 sections covering: past and present perspectives, risk assessment, laboratory testing of ingredients, evaluation guidelines, computer modeling of risk assessment, assessing microbial safety in food, impact of diet, and evaluation of specific foods. Both animal and clinical testing guidelines and safety issues are discussed, along with potential ethical, legal, and regulatory consequences.

Chemiluminescence Immunoassay. By I. Weeks. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1992. 294 pp. Price: U.S. $151.50/Dfl. 295.00. ISBN 0-444-89035-1.

Chemiluminescence immunoassay is now established as one of the best alternatives to conventional radioimmunoassay for the quantitation of low concentrations of analytes in complex samples. During the last 2 decades, the technology has evolved into analytical procedures whose performance far exceeds that of immunoassays based on the use of radioactive labels. Without the constraints of radioactivity, the scope of this type of analytical procedure has widened beyond the confines of the specialist clinical chemistry laboratory to other disciplines such as microbiology, veterinary medicine, agriculture, food, and environmental testing. Overviews are presented of chemiluminescence and immunoassay with the requirements for interfacing chemiluminescent and immunochemical reactions. The possible ways of configuring chemiluminescence immunoassays are described. State-of-the-art chemiluminescence immunoassay systems are covered in detail together with those systems that are commerically available.

New Products

Ion Chromatography Standards

A new line of ion chromatography calibration standards are manufactured from high purity raw materials (typically 99.999% or higher) and are checked against the corresponding National Institute of Standards and Technology SRMs. This new product line includes single ion concentrates at 1000 µg/mL concentration, a selection of 8 stock mixed ion standards and custom single or mixed ion solutions, made to specification. All IC standards are supplied in 100 mL bottles with a full NIST-traceable certificate of analysis and a guarantee of customer satisfaction. VHG Labs, Inc.
Circle No. 325 on reader service card.

Four Channel Portable Gas Detector

The Triple Plus is a 4 channel unit that can simultaneously monitor the atmosphere for flammable gases, toxic gases, and oxygen levels. Alarms are triggered if a dangerous concentration of gas is detected. The detector can be fitted with any combination of up to 4 catalytic, thermal conductivity, or electrochemical sensors such as hydrogen sulfide, hydrogen cyanide, hydrogen chloride, carbon monoxide, chlorine, sulfur dioxide, or nitrogen dioxide. Gases can be monitored instantaneously or as time weighted averages by a single sensor. CEA Instruments, Inc.
Circle No. 326 on reader service card.

High-Pressure Liquid Chromatograph Pump Seals

The new EnerSeal HPLC pump seal's patented design includes an open cavity that minimizes pressure fluctuations during pump operation to ensure precise readouts. The metal-free pump seal is said to prevent leakage at pressures to as high as 8500 psi, and at flow rates from as low as 0.1 mL/min to as high as 32 mL/min. The seal is composed of a UHMW-PE blend, the only material registered with the U.S. Food and Drug Administration for use in clinical processes. This polymer blend is highly friction-resistant, which extends seal life, and is impervious to degradation from the materials under test, which prevents system contamination during pump operations. Advanced Products Co.
Circle No. 327 on reader service card.

Moisture Determination Balance for Routine or Advanced Analysis

The Ohaus MB200 is designed for quick, automatic determination of dry weight or moisture content of processed foods, adhesives, grains, chemicals, pharmaceuticals, and many more materials. With all functions user-selectable from the front panel, the MB200 combines many features including an auto-dry mode and a reliable digital timer to signal when a test cycle has ended with locked in results. A multi-temperature feature allows programming of up to 3 different temperatures during a single cycle. Ohaus Corp.
Circle No. 328 on reader service card.

NIST/EPA/NIH Mass Spectral Database—PC Version

A major update of the PC version of the NIST/EPA/NIH mass spectral database has been released. Produced by the U.S. National Institute of Standards and Technology, the new version contains 9000 new spectra, an increase of over 16%, which takes the total to over 63 000 spectra. This is the only publicly available mass spectral database to consist almost entirely of complete mass spectra. In addition, virtually all of the spectra will have associated chemical structures. These are displayed on the hits screen and with each individual spectrum and can be printed separately. HD Science Ltd.
Circle No. 329 on reader service card.

Fully Integrated System for Cyanide Distillation

The Lab-Crest Midi cyanide system is a fully integrated system for cyanide distillation. The system permits 10 simultaneous distillations in as little as 1/5th the space needed by conventional distillation apparatus. The system conforms to the U.S. Environmental Protection Agency Method 335.2 CLP-M. Fully self-contained, the system is easy to handle, clean, store, and set up. It significantly reduces the amount of reagents used. The borosilicate glass distillation apparatus is integrated with a heater, timer, glassware holder, water and vacuum manifolds, controls, tubing, and quick-disconnect couplings for a compact, complete setup that saves valuable bench space and technician time. The 10-position aluminum heat block is coated with Teflon and the aluminum case is coated with a baked epoxy finish for corrosion resistance. Andrews Glass Co.
Circle No. 330 on reader service card.

Ph Monitoring System Features Hach One Technology

Based on Hach Company's Hach One electrode technology, the EC100 process pH monitoring system is ideal for monitoring and controlling pH in many industrial and municipal applications. The system components include the Series EC100 controller, a Hach One process pH electrode assembly, and a reference electrolyte pump. The electrode assembly features a reference electrode design that replaces conventional porous junctions with a free-flowing, non-clogging liquid/liquid interface. Because there is no porous material to

clog, free electrolyte flow is assured and the electrode lasts longer because it cannot become fouled. Hach Co.
Circle No. 331 on reader service card.

HPLC Sample Injectors

Bio-Rad's sample injectors are 6-port rotary valves with external sample loops, which operate at pressures up to 7000 psi. The injectors use a polished flat stainless steel stator with drilled flow passages and threaded ports for tubing connections. The Model 7010 sample injector injects sample volumes of 10 μL or larger with a sample loop. This injector provides superior volumetric reproducibility by completely filling the sample loop, and is ideal for routine analysis. For applications in which only small quantities of sample are available, the Model 7012 loop filler port permits loading the sample loop by conventional microliter syringes to minimize sample waste. The Model 7125 syringe loading sample injector is a modification of the Model 7010 sample injector in which loading of the loop is accomplished with a syringe through a needle port in the valve rotor. This design allows 2 injection methods: complete loop filling for reproducibility, as in the Model 7010 sample injector; or partial loop filling for no sample loss, using a special syringe. The Model 7125 injector is suitable for routine assays, as well as for methods development. Both injectors are supplied with a 10 μL sample loop. Other sample loop sizes are available. Bio-Rad Laboratories.
Circle No. 332 on reader service card.

20-Bit, Fast, Cost Effective Chromatography Board

The DT2804 is a high resolution (20-bit), fast (75 Hz) data acquisition board for capillary gas chromatography, capillary zone electrophoresis, and liquid chromatography applications. One integrator with 4 time-base independent inputs can sample 4 different detectors from up to 4 chromatographs. Command-driven microprocessor design supports background operation. An RS-232 interface permits operation from a remote host. GLOBAL LAB Data Acquisition Library, a Windows-compatible DLL, is available free with the board. Support is also available from industry-standard application software. Data Translation.
Circle No. 333 on reader service card.

Micro-Robotic Sample Processor

A new micro-robotic sample processor, the AS3000, is optimized for microliter sample preparation procedures and injection onto an HPLC. This new autosampler product line provides automated micro-volume sample preparation that significantly reduces solvent waste and eliminates handling of toxic solvents. The complete automation of sample preparation also increases sample throughput and improves accuracy and precision, especially when working with microliter volumes. The AS3000 is designed with a patented built-in vortex mixer/heater. This vortex mixer/heater replicates the function of normal laboratory vortex mixers/heaters, thus opening opportunities for the automation of new procedures. Spectra-Physics.
Circle No. 334 on reader service cards.

Buffered Listeria Enrichment Broth

A selective enrichment medium for the detection of *Listeria monocytogenes* has been introduced by Unipath. The Oxoid Buffered Listeria Enrichment Broth is intended for use with samples of fermented products, and as an alternate method for the enrichment of environmental samples. The addition of buffers (potassium dihydrogen orthophosphate and disodium hydrogen orthophosphate) enhances the enrichment of *Listeria* species when used with fermented products. The Buffered Listeria Enrichment Broth base is available in 500 gm containers and the Listeria Selective Enrichment Supplement package is sufficient to supplement 5 L of medium. Unipath Co.
Circle No. 335 on reader service card.

TracePur Plus High Purity Acids

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DISCUSSIONS IN ANALYTICAL CHEMISTRY

Analytical Supercritical Fluid Extraction: Current Trends and Future Vistas

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Current Trends

Supercritical fluids have been used as unique solvents in a number of analytical techniques (1), such as nuclear magnetic resonance spectroscopy (2), thin-layer chromatography (TLC) (3), and field flow fractionation (4). The analytical chemist, however, normally associates the prefix "supercritical fluid" with chromatographic or extraction methodologies that have been extensively developed within the past 15 years. The "renaissance" of supercritical fluid chromatography (SFC) occurred in the mid-1970s, largely as a result of improvements in injection and pumping devices, enhanced column efficiencies, and the refinement of transport mechanisms to deliver the separated solutes to modified gas (GC) and liquid chromatographic (LC) detectors (5). By contrast, supercritical fluid extraction (SFE), despite a long history as a physicochemical phenomenon (6) and a recent plethora of applications in chemical engineering (7), has developed as an analytical technique only since the mid-1980s and is presently in an "evolutionary" state.

Despite the early pioneering research of Stahl in coupling SFE with TLC (8), activity in "analytical" SFE remained limited until it was introduced as a pendant technique coupled to SFC. However, this tandem methodology severely limited the sample size that could be extracted with these dense fluids and placed a dual burden on the analyst to optimize both SFE and SFC simultaneously. Because the future of SFC was unknown at the time, commercial instrument manufacturers opted to scale extraction cells to be compatible with the fluid volume and delivery rate capabilities of the syringe pumps used in SFC. Indeed, many of the early extraction cells consisted of modified LC guard cartridges, which were eventually replaced by cell designs that could be "finger-tightened" to withstand extraction pressures up to 10 000 psi.

The development of analytical SFE has also been somewhat hampered by a lack of theoretical guidelines that can be applied to the diverse array of sample types and matrixes encountered by the analyst. Nevertheless, a number of theoretical concepts, ranging from the "solubility parameter theory" (9) to the "hot ball" kinetic model (10), have been applied to optimize SFE conditions. The limitations of such theoretical models, however, become apparent in the actual practice of SFE, as demonstrated by the difference in analyte solubility in a supercritical fluid and its extractability from a specific matrix. For the moment, therefore, experimental optimization of the extraction conditions appears to be the surest way of attaining high analyte recoveries.

The current practice of analytical SFE is divided between "off-line" and "on-line" methods, despite their common physicochemical basis. Such definitions refer to the mechanism of conducting the extraction. The on-line methods are usually combinations of SFE with ancillary techniques such as GC, SFC, LC, or gel permeation chromatography (GPC). Off-line SFE, the current method in vogue, offers more flexibility with respect to extracting different sample sizes and types, as well as in the choice of the final analytical method. Nevertheless, the selection of an SFE method should be based on the problem facing the analyst.

To date, carbon dioxide has been by far the supercritical fluid of choice for analytical SFE (11). The selection of CO_2 was initially based on its widespread use in SFC, its low critical temperature (31°C), and the high degree of nonideality that the gas exhibits even at relatively low levels of compression. Moreover, recent concerns over environmental pollution, the exposure of laboratory workers to noxious solvents, and the disposal costs associated with organic solvents have placed analytical SFE using CO_2 in a different light. The suggested use of such an innocuous solvent as a substitute for organic solvents has been met with skepticism from some scientists. However, supercritical carbon dioxide (SC-CO_2) has proved to be a convenient solvent, whether used as a medium for conducting "Soxhlet-type" extractions of lipid material in various sample matrixes (12) or for extracting ultralow level trace analytes, such as pesticide or drug moieties, from an assortment of sample types (13).

In general, SC-CO_2 mimics the solvent behavior of nonpolar to moderately polar solvents (14). The dissolution power of a supercritical fluid is exponentially proportional to its fluid density; therefore, rapid and exhaustive extractions are best

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handled by conducting the extractions at high pressures. Extraction selectivity for a particular species in SFE is achieved by operating at a lower extraction pressure, but usually at the expense of solute solubility. However, with respect to trace analysis, many analytes, even extremely polar moieties, can be readily dissolved in SC-CO_2 at the ppm or ppb level. Neat SC-CO_2 should not be perceived as a "magic solvent," capable of extracting only the desired target analytes. Coextractives will frequently dissolve in supercritical fluid solvents, just as in liquid solvent extraction. Selectivity can be enhanced for an analyte, or a particular class of analytes (15), by incorporating adsorbents, either in situ or after the initial extraction stage (16).

SFE has been clearly demonstrated to efficiently extract many pesticides and specific drug moieties. Much of the evidence supporting this conclusion has come from extractions performed on spiked sample matrixes (17). However, recent reports of results obtained on samples containing incurred residues (18, 19) confirm the efficacy of SC-CO_2 as an extraction solvent for these analytes. Polar analytes, such as antibiotics, remain a challenge to the analyst because of their lower solubility in SC-CO_2 and their partitioning equilibria, which favor an aqueous medium. However, a number of extraction options remain to be explored with respect to these polar solutes, including the addition of low levels of organic cosolvents or special additives to the supercritical fluid. An impressive range of solubility enhancements for polar solutes in these additive/cosolvent-supercritical fluid systems was recorded (20), and the amount of cosolvent required is still considerably below the volumes required in conventional liquid-based extractions.

The coupled or on-line SFE methodologies mentioned previously appear to have special applications and problems connected with their use. On-line SFE can truly be regarded as a "micro SFE" technique, because the sample sizes used are frequently small (mg level) to avoid solute overload on microbore chromatographic columns. These coupled methods have proved to be of particular value in characterizing small samples such as single seeds, fibers, and live insects. However, considerable skill is required of the analyst to produce uniform and consistent extraction results. On-line SFE tends to be prone to contamination, and the diminutive scale of the technique raises questions about sample uniformity. In addition, procedures for concentrating extracts, such as cryofocusing or sorbent trapping, which are frequently used in on-line SFE/SFC methods, are still poorly understood and are capable of introducing bias in the analytical result.

Current trends in analytical SFE are diverse and worthy of comment. The recent introduction of instrumentation capable of performing extractions on larger and more representative samples is one current trend. As a result, instrumentation manufacturers have had to consider the design of supercritical fluid delivery systems with respect to higher fluid flow rates and extraction pressures. Likewise, the development of multi-sample extractors (21, 22) for the simultaneous processing of large numbers of samples has further catalyzed the creation of new instrumentation. One specialized application of analytical SFE, the determination of fat levels in food products, requires instrumentation capable of producing very high extraction pressures and fluid flow rates. Optimization of the extraction conditions for removing lipid moieties (23) results in the efficient and rapid removal of fats by SC-CO_2 in 15–20 min (24).

The purity level of extraction fluid such as CO_2 has always been suspect, particularly when SFE in either the off- or on-line mode is used in conjunction with ultrasensitive, element-specific detectors. To cite a specific example, the use of off-line SFE in conjunction with electron capture detection, using impure grades of CO_2, will limit the detectability of organochlorine pesticides below the sub-ppm level. Commercial gas manufacturers have recently responded to such needs by producing ultrahigh purity CO_2 in which the total impurity level is in the range of 10 ppt as determined by an electron capture detector (25, 26). These grades of extraction fluids can be expensive for use of exhaustive, high pressure SFE with large samples, suggesting that sorbent-based gas purification systems attached to the extractor modules will need to be developed in the near future. Hence, even industrial grades of CO_2 will have a place in analytical SFE, particularly in the routine determination of the fat content of samples.

The influence of the sample matrix on SFE results was noted by one of the authors (27). Control of sample matrix effects is critical in SFE to limit coextractives, moderate the influence of moisture, and improve the efficiency of the extraction. Recent studies have shown that the addition of both inert and active sorbents to the sample matrix can improve the efficiency of SFE (28). Extractions from difficult sample matrixes, such as soils, can be improved by adding various cosolvents or by using other supercritical fluids, such as nitrous oxide (29, 30).

Future Vistas

What does the future hold for analytical SFE? We believe that the optimal SFE system has yet to be created. Extraction systems need to be developed that offer the flexibility of operating at both higher and lower pressure ranges. Current instrumentation has reached the 10 000 psi level, but theory suggests that many useful extractions can be conducted at higher temperatures and pressures. These conditions will certainly increase the potential molecular weight range of nonthermally labile solutes that can be extracted, but instrumentation must be constructed that is capable of maintaining the proper fluid densities at elevated temperatures. Likewise, SFE is an excellent technique for examining volatile components because the extractions can be conducted at relatively low temperatures and in a nonoxidative environment. These target analytes can best be extracted at pressures that are very low by conventional SFE standards. However, to date most extraction systems offer limited control at the lower extraction pressures required for analysis of volatiles. Certainly, SFE is a viable alternative to headspace techniques, which depend on thermal energy to volatilize analytes; hence, the authors can envisage a bright future for SFE in sensory analysis problems.

Postextraction fractionation will play an increasingly important role in the future of SFE. As alluded to earlier, target

analytes such as pesticides and drugs can be selectively fractionated from unwanted coextractives by using selective sorbents packed in the extraction vessel or in individual vessels downstream from the extraction stage. Such crude fractionations may be viewed as a simple form of normal-phase chromatography when SC-CO_2 is used as the eluant. As such, the analyst should be able to apply LC principles to design the most appropriate supercritical fractionation system. Extraction of unwanted materials by SFE, a form of "inverse" SFE, falls within the context of supercritical fluid fractionations. This form of sample cleanup was already demonstrated on an engineering scale (31) and offers the possibility of isolating analytes from interfering components.

The advantages and disadvantages of coupling SFE with other analytical techniques have already been noted. Several detection and identification schemes coupling mass spectrometry (MS) and Fourier-transform infrared spectroscopy (FTIR) were published (32, 33), but thus far have not found widespread use by the analytical community. Undoubtedly, further research will result in the adoption of some of these hybrid techniques by the analytical chemist. To date, most of the coupled technologies have used rather sophisticated instruments or detectors with SFE. However, a relatively untouched area is the coupling of SFE with simple chemical tests or techniques. One tandem method that was recently explored is the coupling of SFE with immunoassays (33) for the rapid assessment of pesticide contamination in meat products. Such a method offers the possibility of implementation on-site, at a processing plant or inspection station. The method can be made to work with a "pumpless" SFE system and introduces only benign SC-CO_2 and water into the environment. Such simplified SFE systems, along with field portable instrumentation, constitute a wave of the future in analytical SFE.

Conclusion

In concluding our overview of analytical SFE, we should like to make several comments about its implementation in regulatory chemistry protocols. No single analytical technique can hope to solve the diversity of sample preparation problems confronting the analyst; however, analytical SFE will eventually take its rightful place among other sample preparation methods. Successful implementation of SFE will require that analysts expand their horizons and trade some of their conventional tools, such as volumetric flasks and beakers, for pressure gauges and extraction cells. At the same time, the proponents of SFE should attempt to integrate the technique into established analytical protocols, thereby facilitating an easy transition for the bench analyst.

Interest in SFE remains high among such agencies as the Agricultural Research Service (ARS), U.S. Food and Drug Administration (FDA), Food Safety and Inspection Service (FSIS), U.S. Environmental Protection Agency (EPA), Federal Grain and Inspection Service (FGIS), Agricultural Marketing Service (AMS), and some state monitoring agencies. A round robin type of study on sediments and dust has been undertaken by the National Institute of Science and Technology (NIST) and EPA laboratories in the hope of ascertaining the reproducibility of the technique between laboratories. ARS and FDA laboratories now have multi-sample extraction equipment, and commercial counterparts are available. SFE in the laboratories of ARS and FDA was shown to yield both complete and reproducible extractions for pesticide residues down to the 0.5 ppb level. A rough analysis of the savings afforded by using SC-CO_2 as the extraction solvent in place of conventional organic solvents used in the PAM procedures indicates a cost savings of 94–97% (34, 35). This does not include the disposal costs of the organic solvents. However, governmental and industrial laboratories must make their needs known to instrumentation companies if the technique is to remain viable.

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ALCOHOLIC BEVERAGES

Liquid Chromatographic Method for Determination of Glycerol in Wine and Grape Juice: Collaborative Study

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An ion-exchange liquid chromatographic method for the determination of glycerol in wine, white grape juice, and pink grape juice was collaboratively studied by 8 laboratories. Eight wine types and 12 juice samples were provided to each collaborator. Using a strong cation column, blind duplicates and standards were analyzed by an external standard method. Separate statistical evaluations were run on wine, white grape juice, and pink grape juice data. The averages of the relative standard deviations for repeatability, excluding outlying results, were 1.25% for the wine samples, 7.32% for the white grape juice samples, and 8.63% for the pink grape juice samples. The averages of the relative standard deviations for reproducibility, excluding outlying results, were 2.79% for the wine samples, 16.97% for the white grape juice samples, and 19.10% for the pink grape juice samples. The method has been adopted first action by AOAC International.

Glycerol in grape juice has been shown to be an indicator of defects in harvested grapes (1). It is also a primary fermentation product in wine. Therefore, a rapid and reliable method for the determination of glycerol in wine and grape juice is desired.

The 2 AOAC methods for glycerol in wines (2) are very time-consuming due to many washing and evaporating steps. In addition, they have been declared "surplus," indicating they are no longer in current use.

An enzymatic method for the analysis of glycerol (3) requires sample preparation and preparation of many reagents.

Liquid chromatography (LC) for analysis of glycerol in wine and must has been investigated and found to be a rapid and reliable alternative method (4) and has the advantage of requiring only membrane filtration for sample preparation.

A modified LC method for glycerol analysis in wine and grape juice was investigated in the collaborative study reported here. This procedure was selected based on familiarity with the *California Department of Food and Agriculture Wine Grape Inspection High Performance Liquid Chromatography Recommendations* (1986).

Collaborative Study

Eight laboratories participated in the collaborative study. Each collaborator was supplied with sterile-filtered, blind duplicate samples and standards. Although collaborators could detect differences in color in the samples, there should have been no bias because there is no established glycerol content for each wine or juice type. The 8 wines (16 samples) were analyzed as a group with an external standard of 7500 mg/L glycerol. The 12 juices (24 samples) were analyzed with a 1000 mg/L glycerol external standard. The white and pink grape juice data were evaluated separately. A linearity range of glycerol standards (Figure 1) was established using 0, 100, 200, 500, 1000, 2000, 5000, 7500, and 10 000 ppm glycerol (in deionized water).

Collaborators were supplied with a typical chromatogram (Figure 2) and a practice sample set along with the procedure. The practice wine sample contained 12% ethanol, 5000 mg/L glucose, 5000 mg/L fructose, 2000 mg/L tartaric acid, 2000 mg/L malic acid, 1000 mg/L succinic acid, 200 mg/L acetic acid, and 7500 mg/L glycerol. The practice juice sample contained 10% glucose, 10% fructose, 500 mg/L potassium metabisulfite, 2000 mg/L tartaric acid, 2000 mg/L malic acid, and 750 mg/L glycerol. Samples were at room temperature before analysis. After standardization of the instrument, the practice samples were injected. Values of 7500 mg/L for wine and 750 mg/L for juice should have been observed. When satisfactory results were obtained, each sample was injected twice, and the results were reported to 3 significant figures. These replications were averaged for the statistical evaluation. Consequently, the statistical calculations for repeatability and reproducibility were based on the mean of duplicate determinations, performed on each of 2 single test results. This aver-

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The report was evaluated and approved by the General Referee, Committee Statistician, and the Committee on Foods II. The method was adopted first action by the Official Methods Board, August 16, 1991, at Phoenix, AZ. Association actions were published in "Changes in Official Methods of Analysis" (1992) *J. AOAC Int.* 75, January/February issue.

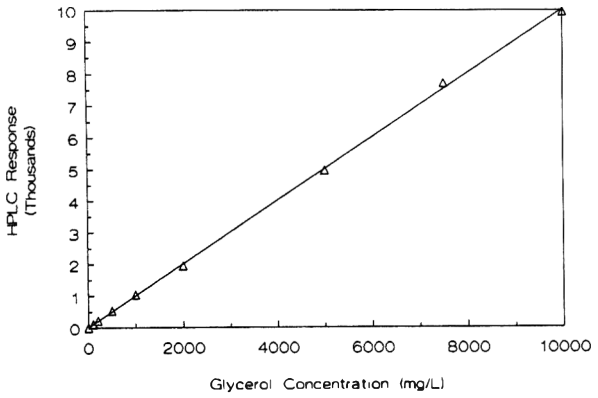

Figure 1. Method linearity.

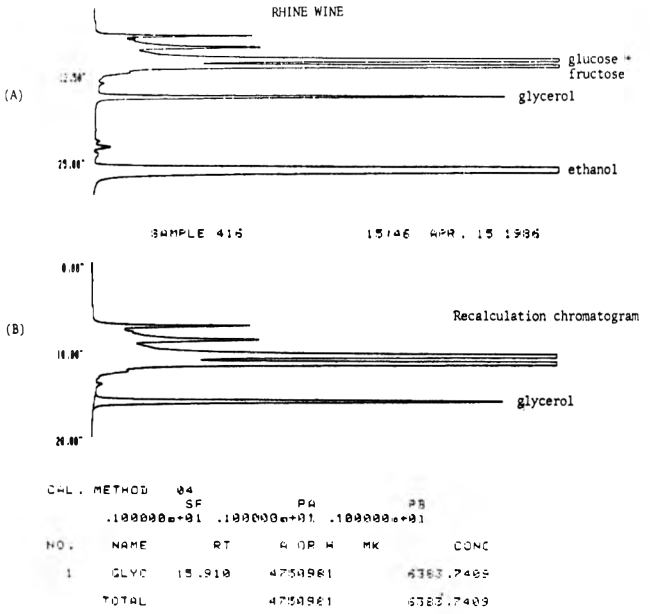

Figure 2. Sample chromatograms: A, real time; B, recalculated chromatogram.

aging may have resulted in lower variances than if variances were calculated from single determinations of each test sample. The data from the wine, white grape juice, and pink grape juice samples were treated as separate experiments statistically.

Collaborators were chosen based on the availability of a suitable LC system and the recommended Bio-Rad HPX87H$^+$ 30 cm column. However, several collaborators achieved acceptable results with other "equivalent" strong cation columns. Collaborators were requested to furnish a chromatogram of wine sample p, noting the type of instrument, integrator, detector, and injector; the settings of the integrator and detector; and all operating conditions and any noteworthy observations. Some collaborators reported problems optimizing integrator parameters and/or instrument conditions.

991.46 Glycerol in Wine and Grape Juice—Liquid Chromatographic Method

First Action 1991

(Applicable to determination of glycerol in wine, and white and pink grape juice)

Method Performance: (Analyte range, 25–10 000 mg/L)

Wine

s_r = 57.96; s_R = 143.69; RSD_r = 1.25%; RSD_R = 2.79%

White grape juice

s_r = 19.29; s_R = 52.55; RSD_r = 7.32%; RSD_R = 16.97%

Pink grape juice

s_r = 31.02; s_R = 55.14; RSD_r = 8.63%; RSD_R = 19.10%

A. Principle

The sample is prepared by membrane filtration. Glycerol in sample is determined by LC, using strong cation column with refractive index detector and integrator, and external standard.

B. Apparatus

(a) *Liquid chromatograph.*—With injector, column heater, refractive index detector, and integrator. Operating conditions: flow rate 0.5 ±0.05 mL/min (glycerol should elute ≥12.0 min); injection volume 10 μL; column temperature 65°C.

(b) *LC column.*—300 mm × 7.8 mm, with strong cation packing (H$^+$ form), theoretical plates, n ≥1500, tailing factor, T ≤1.5 calculated for glycerol (Bio-Rad HPX87H$^+$, No. 125-0740, with n = 2800, T ca 1.0, or equivalent).

(c) *Guard column.*—Deashing system, to remove inorganics (strong cation resin H$^+$; strong anion resin OH$^-$). If LC system (guard and LC column) tailing factor for glycerol >1.5, replace guard column. (Bio-Rad, number 125–0118, or equivalent).

(d) *Membrane filters.*—Disposable, mini-filters with Luer-Lok hub, 0.45 μm (3 cm diam.), attached to 5 mL glass or plastic syringe.

C. Reagents

(a) *Glycerol.*—99.5%.

(b) *Standard solution for wine.*—7500 mg/L glycerol (external standard concentrate) in distilled, deionized H_2O.

(c) *Standard solution for grape juice.*—1000 mg/L glycerol (external standard concentrate) in distilled, deionized H_2O.

(d) *Mobile phase.*—H_2O, LC grade (or filter deionized H_2O through 0.45 μm membrane filter), degas by boiling or helium sparging.

D. Sample Preparation

Filter juice or any unclarified product through 0.45 μm membrane filter.

Table 1. Individual collaborative results for study of glycerol in wine (mg glycerol/L)[a]

	Sample															
	Chablis		Rose		Heavy burgundy		Rhine		Burgundy		French colombard		Chardonnay		Apple	
Laboratory	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
1	5286	5397	6358	6341	8017	8244	4992	4802	7531	7417	5100	5079	6500	6474	3901	3801
	5349	5364	6374	6380	8195	8193	4910	4889	7479	7443	5060	5102	6483	6391	3848	3720
2	5296	5279	6312	6775	8337	8168	4900	4923	7477	7749	5122	5199	6493	6659	3825	3822
	5257	5241	6278	6286	8121	8123	4879	4869	7410	7480	5115	5141	6447	6469	3821	3790
3	5098	5203	6325	6402	8110	8150	4889	4745	7494	7563	5163	4963	6473	6504	3797	3853
	5120	5193	6327	6398	8134	8138	4885	4782	7527	7538	5133	5036	6474	6579	3785	3793
4	5460	5452	6403	6442	7967	8111	4989	4970	7609	6047[b]	4996	5066	6066	5900	3900	3123[b]
	5473	5438	6519	6362	8163	8188	4936	4983	7518	5985[b]	5065	5227	6150	5890	3891	2938[b]
5	5399	5431	6627	6241	8542	8599	5266	5113	7611	7690	4896	5232	6669	6436	4324	4559
	5352	5302	6484	6408	8301	8283	5142	5100	7292	7529	5423	4987	6844	6919	4293	4481
6	5325	5304	6427	6410	8197	8259	4956	4969	7523	7508	5158	5182	6474	6472	3845	3826
	5315	5310	6427	6418	8216	8267	4953	4970	7500	7517	5165	5173	6471	6468	3851	3841
7	5226	5306	6106	6218	7891	7853	4733	4812	7104	7214	5044	4907	6406	6293	6312[b]	4549
	5214	5176	6153	6301	7862	7752	4696	4901	7232	6997	5028	4937	6162	6217	6279[b]	4546
8	5417	5365	6317	6341	8185	8147	4936	4786	7570	7442	5067	5234	6463	6510	3911	3780
	5519	5438	6350	6315	8176	8148	4922	4791	7520	7409	5031	5243	6433	6553	3867	3796
$\bar{X}$	5322		6370		8157		4918		7373	7463[b]	5102		6429		4052	3965[b]
s_r	33.30		91.82		48.34		69.04		393.25	75.41[b]	74.97		69.09		492.01	76.08[b]
s_R	105.11		103.51		166.60		124.36		393.25	149.04[b]	80.36		216.57		703.32	283.97[b]
RSD_r	0.63		1.44		0.59		1.40		5.33	1.47[b]	1.47		1.07		12.14	1.92[b]
RSD_R	1.98		1.63		2.04		2.53		5.33	2.00[b]	1.57		3.37		17.36	7.16[b]

[a] Each result listed is the mean of duplicate determinations of each blind duplicate sample.
[b] Excluded from statistical calculations.

Table 2. Individual collaborative results for study of glycerol in grape juice (mg glycerol/L)[a]

	White grape juice, concn, ppm																							
	0				100				200				500				1000				2000			
Laboratory	a		b		a		b		a		b		a		b		a		b		a		b	
1	65	64	57	53	164	167	157	160	273	273	239	240	549	562	562	558	730	735	735	745	2081	1990	2011	2011
2	52	35	59	63	139	136	134	137	230	235	243	249	534	522	531	529	995	1036	908	941	2015	2010	2007	2003
3	47	48	49	49	140	149	139	145	241	247	249	261	524	523	529	529	1042	1045	958	1023	1978	1975	1982	1985
4[b]	0	0	0	0	0	0	0	0	525	520	267	269	408	412	629	635	1047	1048	1039	1049	1830	1800	1735	1710
5	0	0	0	0	166	148	0[b]	0[b]	253	233	273	238	517	514	542	521	1061	1062	1054	1061	1834	1762	1930	1776
6	48	48	49	42	156	147	145	143	247	230	293	303	531	528	527	527	1064	1071	1065	1069	2021	2028	2024	2028
7	80	79	69	66	181	185	202	197	309	295	312	330	587	572	591	591	1083	1085	1070	1082	2007	2047	1974	1982
8	76	62	91	110	144	146	222	147	356	258	334	335	581	676	595	530	1091	1105	1083	1098	1948	2020	1977	1925
$\bar{X}$	52				146		158[b]		272				549				1003				1976			
s_r	10.49				43.59		12.74[b]		22.85				18.53				28.39				22.71			
s_R	27.90				46.57		20.66[b]		35.30				33.12				126.78				71.55			
RSD_r	20.11				29.80		8.09[b]		8.39				3.37				2.83				1.15			
RSD_R	53.47				31.83		13.11[b]		12.96				6.03				12.63				3.62			
	Pink grape juice, concn, ppm																							
1	77	79	76	73	181	179	190	187	281	286	214	257	594	589	584	562	895	919	860	885	2097	2141	2013	2033
2	69	65	47	53	151	142	152	148	258	251	249	241	555	557	547	560	935	973	1112	971	2050	2025	2033	2048
3	63	66	56	62	156	158	156	157	260	265	244	247	538	541	534	541	1050	1059	966	1075	2023	2037	2043	2043
4[b]	0	0	0	0	860	835	45	50	110	115	205	271	460	453	405	400	1055	1143	1053	1049	189	191	1795	1781
5	0	0	0	0	283[b]	343[b]	166	193	308	276	274	205	466	445	438	498	1063	1054	1058	1091	1763	1839	1850	2071
6	63	62	61	62	170	177	166	161	255	254	294	350	557	554	558	553	1048	1094	1076	1060	2036	2027	2031	2030
7	104	87	99	90	215	205	217	198	319	316	335	0	606	590	558	618	1107	1065	1088	1118	2043	2043	1985	1997
8	69	164	92	102	169	208	159	203	311	523	342	0	576	553	590	617	1071	1060	1067	1110	1940	1940	1939	2005
$\bar{X}$	66				185		175[b]		265				553				1033				2004			
s_r	7.14				35.93		4.54[b]		81.51				12.33				28.14				52.48			
s_R	34.65				42.05		21.12[b]		81.51				45.50				73.41				74.64			
RSD_r	10.86				19.38		2.59[b]		30.78				2.23				2.72				2.62			
RSD_R	52.71				22.68		12.03[b]		30.78				8.23				7.10				3.72			

[a] Each result listed is the mean of duplicate determinations of each blind duplicate sample.
[b] Excluded from statistical calculations.

E. Determination

Inject standard until reproducibility of <2% is obtained for 5 successive injections. Use series of 3 injections to establish average calibration for standard.

Inject sample. Calculate mg/L glycerol in sample as follows (or by integrating microprocessor):

$$\text{Glycerol, mg/L} = (AC/AC') \times C'$$

where AC and AC' = peak area for sample and standard, respectively; and C' = glycerol concentration in standard.

Ref.: JAOAC 75, May/June issue (1992)

CAS-56-81-5 (glycerol)

Results

Data and statistical results for wine, white grape juice, and pink grape juice are listed in Tables 1 and 2. The Cochran test (5) disqualified Laboratory 4 for both white and pink grape juice data, so these data were eliminated in subsequent statistical analyses. Although variation between replicate samples was low for Laboratory 4, blind duplicate samples showed a large variance. This collaborator reported difficulty with the equipment and sample analysis. The Cochran test also identified outliers among individual results, and the statistical evaluation in Tables 1 and 2 is shown with and without these outliers.

In the wine experiment, the relative standard deviations for repeatability (RSD_r) and reproducibility (RSD_R) are low except for the apple wine sample, and those values become low when outliers are omitted (Table 1).

In the white grape juice experiment, the RSD_r and RSD_R are slightly higher, especially at the lower glycerol concentrations (Table 2). These results appear to agree with work by Horwitz et al. (6), which reports an inverse relationship between the coefficient of variation and sample concentration.

The pink grape juice experiment results are similar to those of the white grape juice experiment (Table 2).

Recommendation

We recommend that the liquid chromatographic method for the determination of glycerol in wine and grape juice be adopted first action.

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C. Nagel, Washington State University, Pullman, WA

M. Salehi, Bronco Wine Co., Ceres, CA

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CEREALS AND CEREAL PRODUCTS

Interlaboratory Study of Decreasing the Number of Standard Points in the Official Iron Standard Curve

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An interlaboratory study was conducted to (*1*) evaluate the effects of reducing the 10 points for the iron standard curve to 5 points in *Official Methods of Analysis* (1990) 15th Ed., secs 944.02A–944.02C(a), and (*2*) compare the levels of iron found in foods by using the 10-point and 5-point standard curves. The 5 points (0.2, 0.6, 1.0, 1.4, and 1.8 μg Fe/mL) were selected by eliminating every other standard point from the 10-point curve after correction for the reagent blank. No differences in the performance parameters between method versions were found when blind duplicate analysis was used to estimate the performance parameters for each sample analyzed.

This study was conducted with the goal of reducing the number of points required to prepare the standard curve for iron in foods, *Official Methods of Analysis* (1990) 15th Ed., sec. **944.02B** (1). The method was established in 1945. Iron (Fe) concentrations were measured by using a 2 in. cell in the neutral wedge photometer (2). With today's modern state-of-the-art spectrophotometers, fewer standard points are required to obtain a linear curve, as demonstrated by extensive observations reported in an in-house publication (3).

The interlaboratory study consisted of 8 duplicate samples analyzed by 7 laboratories. Six products were analyzed on separate days; they consisted of milk-based infant formula powder (low Fe), soy-based infant formula powder (Fe-fortified), enriched bread, whole wheat flour, and fortified and nonfortified cereals. Enriched macaroni and enriched egg noodles were used as blind duplicates and were assayed on the same day. Although 7 laboratories participated in this study, the results from 2 collaborating laboratories were discarded because of failure to follow directions.

Interlaboratory Study

No sample preparation, other than thorough mixing, was required for the infant formulas and the whole wheat flour. The enriched bread was air-dried for 2 days and ground in a Brinkmann mill with a 2 mm sieve. The cereals, enriched egg noodles, and enriched macaroni were ground in a Brinkmann mill with a 2 mm sieve. All samples were thoroughly mixed and then dispensed into individually identified small screw-cap plastic bottles. Each sample plus a practice sample was identified for the appropriate day to be assayed. The analysts were instructed to refrigerate the samples upon arrival and to allow them to come to room temperature before they were opened for analysis. Each analyst was given sample and standard data sheets to record the raw data and was requested to submit these completed data sheets and all instrument-generated graphs and charts.

The method used in this study is described in *Official Methods of Analysis* (1990) 15th Ed., secs **944.02–944.02C(a)**, with the following changes: (*1*) a 5.0 g sample was used, instead of the stated 10.0 g; (*2*) the first dilution was filtered, if necessary, through ashless filter paper and the first 15–20 mL filtrate was discarded; and (*3*) after the addition of the hydroxylamine HCl reagent, there was a 5 min wait before the acetate buffer solution (**944.02C**) was added.

The analysts were instructed to prepare the iron solution for the standard curve according to sec. **944.02A (c)**(1); use the *o*-phenanthroline, hydroxylamine HCl, and iron wire provided to them; analyze the designated samples on separate days; increase the dilution if a sample absorbance reading exceeded the highest standard point and maintain the required HCl concentration as well as the amounts of reagents in the rediluted sample solution; and finally, enter the raw data for each sample, standard, and blank reading on the data sheets provided.

Results and Discussion

The sample data were statistically evaluated according to the *Guidelines for Collaborative Procedures* (4) and the SAS analysis of variance (5). The data were treated by using the Day I and Day II values as replicates for all samples except enriched macaroni and enriched egg noodles, which were replicated on the same day. The day-to-day variability for a given sample was not differentiated from variability between same-day replicates of a sample. Table 1 summarizes the performance

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Table 1. Comparison of the performance parameters of iron content between the 5-point and 10-point versions

Product	Standard curve version	Mean, µg Fe/100 g	s_r[a]	s_R	RSD_r	RSD_R
Milk-based infant formula[b]	5	1.48	0.13	0.21	8.48	14.20
	10	1.47	0.13	0.19	8.96	13.21
Soy-based infant formula[c]	5	9.64	0.43	0.59	4.43	6.13
	10	9.62	0.44	0.58	4.61	5.99
Nonfortified cereal	5	5.95	0.18	0.26	3.08	4.41
	10	5.95	0.19	0.25	3.20	4.23
Fortified cereal	5	22.30	1.24	1.24	5.55	5.55
	10	22.34	1.18	1.18	5.29	5.29
Enriched bread	5	5.25	0.64	0.64	12.14	12.14
	10	5.25	0.63	0.63	12.09	12.09
Whole wheat flour	5	4.77	0.16	0.40	3.43	8.39
	10	4.76	0.15	0.39	3.21	8.18
Enriched macaroni	5	4.54	0.12	0.27	2.71	5.96
	10	4.53	0.14	0.27	3.10	5.87
Enriched egg noodles	5	4.10	0.08	0.15	2.05	3.54
	10	4.10	0.09	0.13	2.10	3.24

[a] s_r = repeatability: within-laboratory precision; s_R = reproducibility: among-laboratories precision; RSD_r = relative standard deviation for repeatability; RSD_R = relative standard deviation for reproducibility.
[b] Powder–low Fe.
[c] Powder–Fe-fortified.

parameters of each version of the method. On the basis of duplicate assays from each of 5 laboratories, the iron results from each version of the method (5-point or 10-point iron standard curve) were statistically analyzed by using a blind duplicate analysis to estimate the performance parameters; they clearly show no significant difference between method versions.

Recommendation

On the basis of the interlaboratory study, the 5-point iron standard curve gives the same precision and accuracy as the currently used 10-point curve and is recommended for adoption as official first action.

The following changes and additions to the method are suggested: (*1*) In the Determination, **944.02C(a)**, paragraph 3, line 2, replace "in few min" with "let stand 5 min." (*2*) Insert a statement concerning the importance of maintaining the HCl concentration throughout the entire dilution scheme, because proper color development is pH-dependent; for example, "If further dilution is required to maintain the sample absorbance reading below the highest standard point on the curve, pipet a smaller aliquot into a 25.0 mL flask, dilute to 10.0 mL with 2% HCl solution, and continue as described in sec. **944.02C**, para. 3."

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CHEMICAL CONTAMINANTS MONITORING

Total Diet Study of Lead and Cadmium in Food Composites: Preliminary Investigations

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In a trial for a comprehensive total diet study, 105 food composites were prepared in the summer of 1985, and another 105 composites in the late fall of 1985. Lead and cadmium were determined in all composites. The mean, median, and range of lead concentrations in the samples were 29.9, 14.7, and 1.42–407 ng/g, respectively. The estimated dietary ingestion of lead by the average adult Canadian was 36.4 μg/day. The mean, median, and range of cadmium concentrations were 13.7, 5.4, and <0.07–297 ng/g. Dietary ingestion of cadmium by adults averaged 14.5 μg/day. Sample planning and contamination control for trace element determinations of the study are discussed.

Total diet surveys are used to analyze a variety of chemical compounds in foods prepared for consumption. These surveys estimate intakes of compounds via the food supply and pinpoint those products that contain higher than average background levels or are nutritionally deficient. As a result of these estimations, the relative safety of the food supply can be assessed, and steps can be taken to improve food quality.

In 1985, the Canadian Health Protection Branch (HPB), as part of an ongoing total diet program, initiated a new survey in which 105 food composites were chosen to represent foods consumed in Canada (1). As part of the preliminary trial for the survey, foods were collected in the summer of 1985 in the National Capital Region. These were processed and combined into 105 composites (Set 1) (1). A second set was then prepared from foods collected in the late fall of 1985 from the same geographic region. The composites were analyzed for selected nutrients, pesticide residues, and contaminants.

Numerous factors go into planning total diet surveys, including what types of products are chosen, how products are prepared, and how closely products represent actual consumption patterns by different age and sex groups (1). In addition to general considerations, however, special attention must be given to the individual nutrients or contaminants scheduled for analysis. To embark on any survey of this scope, the expertise of analytical chemists, nutritionists, and others must be included in the preliminary planning stages. For example, when foods are stored, they must not be subject to contamination or lose their nutrient value before they are analyzed. Thus, care must be taken during sample collection and processing, proper containers must be used, and the appropriate temperature and duration of storage must be chosen. Also, subdivision of some food composites into constituent foods can be planned to better estimate the contribution from suspected sources of contamination.

Lead and cadmium were among the contaminants determined in foods prepared during this trial. This paper describes how the total diet survey was designed to include lead and cadmium in the survey and presents the analytical results.

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Experimental

Sample choice.—Canned and/or raw (fresh or frozen) foods, purchased at the retail level in Ottawa, were prepared for consumption as they would be in a home setting. The prepared foods were then combined into 105 composites, homogenized, and frozen until analysis. The whole process was repeated again after 6 months.

In some cases, to obtain information regarding the effects of processing, cooking, etc., samples of the unprepared foods were also analyzed. For example, both raw and cooked meats were analyzed, although the food composites included only the cooked meats. Also, the canned constituents of some composites were analyzed separately to determine the potential contribution of lead-soldered cans to the lead level.

Sample processing.—Stainless steel utensils and blenders with glass containers were used throughout to avoid contaminating the foods with lead and cadmium. Instructions were given to avoid powdered cleansers, gloves with talcum powder, and apparatus in which lead-soldered parts might contact the foods. Bench tops, tables, and cutting boards were washed thoroughly with tap water between samples to avoid cross contamination.

Facilities were not available for preparing large quantities of deionized water, and a decision was made to use laboratory (community) tap water for washing and processing the foods. Previous analyses of this water revealed lead levels usually below 2 ng/mL, with a maximum concentration of 5 ng/mL. Because no detergents were used (to avoid contamination of

Table 1. Quality control results for cadmium

Analyzed batch	Blank spike rec., %[a]		Sample spike rec., %[a]		Detection limit, ng/g	
	Set 1[b]	Set 2	Set 1	Set 2	Set 1	Set 2
1[c]	105	101	95	78	0.38	0.36
2	104	95	59	94	0.43	0.28
3	104	89	108	90	0.33	0.66
4	97	87	124	88	0.08	0.61
5	87	99	80	99	0.37	0.16
6	92	94	72	94	0.20	0.17
7	93	94	—	68	0.33	0.07
8	94	94	—	96	0.07	0.07
9	94	86	92	82	0.01	0.14
10	99	92	99	91	0.05	0.06
11	91	—	94	—	0.03	—
Av.	96	93	91	88	0.21	0.26
Av., both sets	94		90		0.23	

[a] 50 ng cadmium was spiked before digestion.
[b] In this and all subsequent tables, the samples collected in the summer of 1985 are designated as Set 1. Those collected in the late fall are designated as Set 2.
[c] The summer samples were analyzed in 11 batches and the fall samples in 10 batches.

the foods with organics, which were analyzed separately), extremely hot water was needed for washing utensils and equipment. This was obtained by installing a separate water-heating tank for the laboratory midway through the preparation of Set 1 samples.

To obtain better homogeneity, food composites 32, 33, 34, 36, and 38 were dried before homogenization. The percentages of moisture content (31, 40, 43, 32, and 24%, respectively) were used to adjust the reported concentrations to a wet weight basis.

Sample storage.—Foods designated for trace element analysis were stored in high-density, large-mouth, polyethylene square bottles, 175 mL capacity, with screw caps. Bottles were prewashed with nitric acid, rinsed with deionized water, dried, and tested for contamination by leaching with 5% nitric acid. Bottles contained no metal liners that could contaminate the samples.

Analytical reagents.—Deionized water (ASTM Type III) was used wherever water was specified.

For lead and cadmium determinations, nitric and perchloric acids were purified by sub-boiling distillation in a quartz still. Lead nitrate (NIST SRM No. 928) and high-purity cadmium metal (m6N, No. 00062, Alfa Products, Danvers, MA 01923) were stock materials used for preparation of standards. Ammo-

Table 2. Quality control results for lead

Analyzed batch	Blank spike rec., %[a]		Sample spike rec., %[a]		Detection limit, ng/g	
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2
1	103	101	106	99	0.3	4.6
2	98	92	93	106	0.8	1.1
3	100	92	109	91	0.3	1.9
4	93	88	96	88	0.6	2.8
5	89	94	85	95	1.3	2.4
6	92	93	89	91	1.7	1.0
7	92	87	87	86	1.3	1.7
8	97	95	—	110	1.0	0.7
9	95	96	—	95	1.6	1.9
10	94	101	86	103	3.2	1.0
11	90	—	88	—	0.1	—
Av.	95	94	93	96	1.1	1.9
Av., both sets	94		95		1.5	

[a] 500 ng lead was spiked before digestion.

Table 3. Cadmium and lead levels in individual composites

Composite	Category and food	Cd, ng/g		Pb, ng/g	
		Set 1	Set 2	Set 1	Set 2
	Milk and dairy products				
1	Milk, whole	0.37	0.40	1.4	2.5
2	Milk, 2% B.F.	0.11	0.22	1.5	1.9
3	Milk, skim	0.16	0.52	1.0	3.3
4	Instant breakfast prepd.	4.13	0.98	8.0	4.7
4A	Instant breakfast dry	50.13	—	48.6	—
5	Cream	0.33	0.23	1.8	2.7
6	Ice cream, mixed	0.84	1.98	12.3	5.2
6A	Ice cream, chocolate	—	—	—	9.9
7	Yogurt, mixed	0.61	0.30	0.7	4.8
7A	Yogurt, plain	0.53	0.31	1.8	3.4
8	Cheese	6.04	0.14	1.7	16.2
9	Cottage cheese	0.21	0.67	0.4	6.5
10	Cheese, processed	24.19	0.27	24.2	17.6
11	Butter	0.68	1.04	< 0.3	6.5
	Meat and poultry				
12	Beef steak, cooked	14.02	0.41	11.4	10.2
12A	Beef steak, raw	26.62	2.84	68.8	16.2
13	Roast beef	2.50	1.97	10.5	16.6
14	Ground beef, cooked	5.68	0.76	41.6	12.4
14A	Ground beef, raw	2.80	0.62	17.1	7.8
15	Pork, cooked	6.37	2.70	273.3	5.3
15A	Pork, raw	4.75	0.41	15.8	4.0
16	Pork, cured	3.13	3.09	156.3	22.5
17	Veal, cooked	1.86	0.97	25.8	7.6
17A	Veal, raw	3.85	2.47	13.3	24.4
18	Lamb, cooked	6.92	1.47	21.2	8.2
18A	Lamb, raw	8.25	0.61	15.2	5.7
19	Poultry, cooked	1.22	1.34	18.6	3.0
19A	Poultry, raw	2.01	1.93	8.8	22.1
20	Eggs	0.89	0.36	3.3	3.5
21	Meat organs	70.65	44.34	76.7	47.9
22	Cold cuts luncheon meats	2.78	1.97	14.2	48.5
23	Luncheon meat, canned	3.06	2.95	47.7	66.8
	Fish				
24	Marine fish, cooked	2.64	4.46	44.7	13.5
24A	Marine fish, raw	79.40	7.56	10.8	12.9
25	Freshwater fish, cooked	7.68	1.24	8.1	4.7
25A	Freshwater fish, raw	1.51	3.72	7.7	20.9
26	Fish, canned	13.78	9.13	168.9	34.1
27	Shellfish	297.40	165.50	298.4	41.0
	Soups				
28	Soups, meat, canned	3.89	1.93	20.2	25.3
29	Soups, pea, canned	8.40	7.98	25.3	42.0
30	Tomato soup, canned	9.42	11.09	39.3	34.2
31	Soups, dehydrated	5.92	1.94	10.4	3.7

Table 3. ***Continued***

Composite	Category and food	Cd, ng/g		Pb, ng/g	
		Set 1	Set 2	Set 1	Set 2
	Bakery goods and cereals				
32	White bread, all	15.68	14.66	30.5	36.7
32A	White bread, no raisins	17.93	14.72	22.4	14.4
33	Bread, whole wheat and rye	18.12	16.20	34.1	20.2
34	Bread rolls and biscuits	14.01	12.38	15.4	14.5
34A	Rolls and buns only	—	12.76	—	13.9
35	Wheat flour	19.83	17.09	5.0	1.6
36	Cake & muffins with raisins	12.17	10.37	36.2	18.9
36A	Cake and muffins	14.06	11.91	21.6	26.2
37	Cookies, all	17.23	15.73	23.1	16.3
37A	Cookies, oatmeal, arrowroot	16.97	11.08	31.8	14.4
37B	Cookies, chocolate chip	23.16	21.41	23.2	21.7
38	Danish and donuts	10.62	8.66	13.3	18.9
39	Crackers	23.36	21.43	14.1	23.1
40	Waffles and pancakes	9.20	9.13	18.6	21.1
41	Cooked wheat cereal	6.03	25.40	4.3	1.7
41A	Cream of wheat, dry	32.11	7.70	3.5	8.6
42	Oatmeal cereal	2.20	3.79	5.1	6.7
42A	Oatmeal cereal, dry	12.43	18.59	9.6	3.1
43	Corn cereal	4.73	3.67	136.9	6.4
44	Wheat and bran cereals	68.50	99.28	92.0	20.0
45	Rice cereal, cooked	20.19	11.47	11.8	9.5
45A	Rice cereal, dry	64.02	22.99	9.5	5.3
46	Apple pie	9.99	7.47	26.2	13.1
47	Pie, others, mix	11.77	7.16	180.8	37.3
47A	Pie, no raisins	5.17	7.99	7.7	19.6
47B	Raisin pie	50.38	8.99	488.2	247.4
48	Pizza	25.38	14.26	31.2	18.9
49	Pasta, canned	16.64	11.31	34.2	7.9
50	Pasta, plain, cooked	41.60	33.59	26.1	14.1
	Vegetables				
51	Corn, raw & canned, cooked	8.02	1.20	36.8	51.8
51A	Corn, raw	2.43	1.86	40.3	181.5
51B	Corn, kernel, canned	10.25	2.85	4.7	5.7
52	Potatoes, raw	46.20	34.52	21.9	7.8
53	Potatoes, baked	94.35	38.43	2547.0	40.6
54	Potatoes, boiled, skins	60.69	31.77	11.5	4.0
55	Potatoes, peeled, boiled	57.67	26.16	3.6	3.8
56	French fries	55.98	52.76	3.3	5.1
57	Potato chips	123.90	122.50	15.1	12.0
58	Cabbage, cooked & coleslaw	7.51	2.55	1137.0	4.6
59	Celery	45.13	64.52	29.9	44.9
60	Peppers, green & red	13.43	9.52	54.7	8.7
61	Lettuce	13.81	39.73	1025.0	2.3
62	Cauliflower, raw & cooked	13.94	9.02	21.1	63.7
62A	Cauliflower, raw	—	9.88	—	1.9
63	Broccoli, raw and cooked	11.29	30.06	35.6	16.0
64	Beans, raw & canned, cooked	7.29	1.72	1689.0	72.6
64A	Beans, raw	4.01	1.90	34.3	13.3
64B	Beans, canned	4.05	0.80	735.7	120.2
65	Peas, raw & canned, cooked	5.83	2.24	32.3	14.7
65A	Peas, raw	11.13	6.26	13.2	9.6

Table 3. ***Continued***

Composite	Category and food	Cd, ng/g		Pb, ng/g	
		Set 1	Set 2	Set 1	Set 2
65B	Peas, canned	11.65	3.19	2371.0	23.9
66	Carrots, raw & canned, cooked	19.68	17.45	50.1	9.7
66A	Carrots, raw and canned	14.05	—	8.0	—
66B	Carrots, raw	11.54	17.79	12.2	9.1
66C	Carrots, canned	9.58	—	115.9	—
67	Onions, cooked and raw	39.34	12.58	7.5	8.1
67A	Onions, raw	—	16.72	—	9.4
68	Turnips, parsnips	—	18.85	—	15.6
69	Tomatoes, raw and cooked	18.79	4.93	2165.0	2.5
69A	Tomatoes, cooked	25.08	6.03	2547.0	1.4
69B	Tomatoes, raw	20.51	5.92	914.1	2.0
70	Tomato juice, canned	6.87	16.77	338.9	24.0
71	Tomatoes, canned	14.72	52.42	935.3	109.4
72	Mushrooms, raw & canned, cooked	11.89	13.12	67.3	44.6
72A	Mushrooms, canned	55.45	14.52	13.5	70.9
72B	Mushrooms, raw	107.60	14.07	59.6	52.9
73	Cucumber, raw, pickled	7.47	5.35	228.2	8.3
73A	Cucumber, raw	7.95	2.98	458.2	8.1
	Fruit and fruit juices				
74	Citrus fruit, raw	0.57	0.18	7.2	18.3
75	Citrus fruit, canned	0.19	< 0.07	165.8	407.4
76	Citrus juice	0.52	< 0.07	5.9	19.7
77	Citrus juice, canned	0.08	< 0.07	82.1	17.8
78	Apples	0.78	0.44	12.8	18.2
79	Apple juice, canned & bottled	0.79	0.55	14.1	24.9
79A	Apple juice, bottled	0.95	2.75	19.9	10.4
79B	Apple juice, canned	0.43	0.57	9.8	23.3
80	Apple sauce, canned & bottled	0.30	0.14	34.4	48.2
80A	Apple sauce, bottled	0.09	< 0.07	5.7	12.7
80B	Apple sauce, canned	0.08	8.43	56.3	102.3
81	Bananas	0.86	0.15	< 1.6	< 1.9
82	Grapes	1.31	—	6.9	—
83	Grape juice, bottled	2.62	0.84	24.7	21.5
84	Peaches, canned and raw	1.45	1.39	177.7	119.7
84A	Peaches, raw	2.95	—	19.8	—
85	Pears, raw and canned	0.80	1.20	140.8	97.6
85A	Pears, canned	1.05	—	162.1	—
85B	Pears, raw	2.22	2.63	15.0	8.6
86	Plums, prunes, dried & canned	6.02	0.82	355.7	206.7
87	Cherries, raw and canned	0.48	—	12.9	—
87A	Cherries, canned	0.57	5.66	88.6	203.8
88	Melons	6.35	14.20	6.7	1.9
89	Strawberries	11.12	17.10	11.4	7.6
90	Blueberries	1.27	0.39	47.2	21.2
91	Pineapple, raw and canned	1.30	1.10	4.1	15.8
91A	Pineapple, fresh	1.68	1.31	< 0.6	15.2
91B	Pineapple, canned	—	0.51	—	31.3
	Fats and oils				
92	Cooking fats & salad oils	0.91	0.66	9.9	3.1
93	Margarine	3.66	0.65	< 0.2	6.0
94	Peanut butter	39.23	48.63	24.5	15.2

Table 3. ***Continued***

Composite	Category and food	Cd, ng/g		Pb, ng/g	
		Set 1	Set 2	Set 1	Set 2
	Sugar and candies				
95	Sugar	0.37	5.03	52.2	< 1.9
96	Syrup	2.96	1.48	132.8	69.0
97	Jams and jellies	5.75	5.63	176.9	19.2
98	Honey	0.69	0.83	22.3	41.8
99	Pudding, canned, mixed, powder, prepared	2.44	0.70	13.3	8.8
99B	Pudding, chocolate powder and canned	3.91	4.48	9.3	30.3
100	Candy, chocolate bars	28.29	16.81	51.6	40.2
101	Candy, other	3.52	0.61	79.4	51.5
	Beverages				
102	Coffee	0.64	0.10	4.1	5.2
103	Tea	0.50	0.45	7.1	9.2
104	Soft drinks	0.57	0.19	2.7	5.1
105	Wine and beer, cans and bottles	1.47	0.62	16.0	76.9
105A	Wine	0.97	0.64	28.5	140.6

nium pyrrolidine dithiocarbamate solution was prepared fresh daily and purified by filtering through a 0.3 μm cellulose acetate filter.

Analytical instrumentation.—A Model 875-ABQ atomic absorption spectrophotometer was used in conjunction with a GTA-95 graphite-furnace atomizer to determine lead and cadmium (Varian Analytical Instruments, Sunnyvale, CA 94034). Plateau-type pyrolytically coated graphite tubes with pyrolytic graphite platforms were purchased from the manufacturer. Lead and cadmium were determined at 283.3 and 228.8 nm under the instrumental conditions described previously (2).

Lead and cadmium methodology.—After a nitric–perchloric acid digestion, the ammonium pyrrolidine dithiocarbamate complexes were coprecipitated with copper and iron carriers and dissolved in nitric acid containing ammonium dihydrogen phosphate modifier (2). The final concentration of ammonium dihydrogen phosphate was 0.1%, a modification of the original method.

Quality control for lead and cadmium.—Quality control was maintained as follows: Each analytical batch included a minimum of 3 reagent blanks used to monitor contamination and estimate detection limits, and the recoveries from 2 spiked reagent blanks and 2 spiked samples were calculated. The method required that the sample concentrations be corrected for the recovery of spikes (500 ng lead and 50 ng cadmium) added to the reagent blanks before digestion to compensate for day-to-day variation of instrumental response to synthetic standards and coprecipitated samples.

Calculations and dietary intake estimations.—To estimate means, medians, and ranges of concentrations, samples with concentrations less than the detection limit of the batch were reported as being at the detection limit. In those instances in which both the constituents of a composite and the composite itself were analyzed, no distinction was made between them, and all concentrations were included when means, medians, and ranges were calculated. Only the composite results were used for estimating dietary ingestion of lead and cadmium.

Food intake data were based on 112 finalized composites (1) and primarily reflect average ingestion by adults and older children of both sexes. Because the categories of muffins, baked beans, raisins, wieners, gelatin dessert, and beets were not separate composites when this preliminary study was conducted, the weights given by Conacher et al. (1) for these categories were added to those given for the following composites: cake and muffins with raisins (No. 36), beans (No. 64), raisin pie (No. 47B), cold cuts and luncheon meats (No. 22), and turnips (No. 68), respectively.

Estimations of trace element intake were based on the sum over all composites of the product of the intake in grams of each composite and its lead or cadmium concentration.

Results and Discussion

Lead and cadmium quality control results.—Set 1 composites were analyzed in 11 analytical batches, and Set 2 composites were analyzed in 10 batches. The average recoveries of cadmium and lead from spiked blanks (duplicates) and samples (duplicates) are listed in Tables 1 and 2 for each analytical batch in both sets.

The recovery of 500 ng lead added to the reagent blanks before digestion averaged 94% and ranged from 87 to 103%. Sample spike recoveries averaged 95% and ranged from 85 to 110%. The spiking level was equivalent to a sample concentration of about 25–500 ng/g, depending on the actual sample weight taken.

Table 4. Cadmium levels in food categories

Category	Description	Set	*n*	Mean, ng/g	Median, ng/g	Range, ng/g
I	Milk and dairy products	1	13	6.79	0.61	0.11–50
		2	12	0.59	0.40	0.14–2.0
II	Meat and poultry	1	18	9.30	3.85	0.89–70.7
		2	18	3.96	1.93	0.36–44.3
III	Fish	1	6	67.07	7.68	1.51–297
		2	6	31.94	4.46	1.24–166
IV	Soups	1	4	6.91	5.92	3.89–9.4
		2	4	5.74	1.94	1.93–11.1
V	Bakery goods and cereals	1	28	20.84	16.97	2.2–69
		2	29	16.59	12.76	3.7–99
VI	Vegetables	1	36	27.20	13.81	2.4–124
		2	37	19.27	13.12	0.8–123
VII	Fruit and fruit juices	1	27	1.73	0.95	0.08–11.12
		2	24	2.53	0.82	< 0.07–17.1
VIII	Fats and oils	1	3	14.60	3.66	0.91–39.2
		2	3	16.65	0.66	0.65–48.6
IX	Sugar and candies	1	8	5.99	2.96	0.37–28.3
		2	8	4.45	1.48	0.61–16.8
X	Beverages	1	5	0.83	0.64	0.5–1.47
		2	5	0.40	0.45	0.1–0.64
All composites		1 + 2	294	13.69	5.35	< 0.07–297
		1	148	16.16	6.35	0.8–297
		2	146	11.20	3.67	< 0.07–166

The average recovery of 50 ng cadmium added to the reagent blanks before digestion was 94% (range 86–105%). Recoveries from spiked samples were less consistent than those obtained for lead, averaging 90% and ranging from 59 to 124%. The potential contribution of sample inhomogeneity to the recoveries was not evaluated. The spiking level for cadmium was equivalent to a sample concentration of about 3–50 ng/g.

The detection limits of the method were defined as 3 times the standard deviation of the replicate blanks within each analytical batch divided by the average sample weight. Detection limits averaged 1.5 ng/g (range 0.1–4.6 ng/g) for lead and 0.23 ng/g (range 0.01–0.66 ng/g) for cadmium.

Cadmium survey results.—Determination of cadmium in the individual samples revealed that only shellfish (No. 27) and potato chips (No. 57) contained levels that consistently exceeded 100 ng/g (Table 3). Chocolate bars (No. 100) had higher cadmium levels than other candy (No. 101). No significant increase in cadmium levels as a result of cooking was found.

A summary of the cadmium levels in the individual food categories showed that fish, bread and cereals, and vegetables contained the highest levels (Table 4).

For cadmium in Set 1, the mean, median, and range of concentrations in all the individual composites were 16, 6.4, and 0.8–297 ng/g, respectively (Table 4). The respective concentrations for Set 2 were 11.2, 3.7, and <0.07–166 ng/g. The means of 16 and 11.2 ng/g for all samples are similar to an average level of 10.8 ng/g found in a 24 h duplicate diet survey (3).

The estimated dietary intake of cadmium by Canadians was 16.8 and 12.2 μg/day for Sets 1 and 2, respectively. The average of 14.5 μg/day agrees well with 13.8 μg/day found in a previous Canadian 24 h duplicate diet survey (3). The average of 14.5 μg/day is equivalent to an intake of 0.21 μg/kg body weight/day, which is about one-fifth of the FAO/WHO provisional tolerable daily intake of cadmium from all sources, i.e., 0.96–1.2 μg/kg/day.

Lead survey results.—Analysis of the individual composites revealed several peculiarities (Tables 3 and 5). Some of the samples in Set 1 (No. 15, 53, 58, 61, 64, 65, 69, 69A, 69B, 73, 73A) had unusually high lead levels (Table 5). This was attributed to "in house" contamination for 2 reasons: First, a second set of samples from Ottawa (Set 2), as well as similar samples from 2 other cities (Sets 3 and 4), had consistently lower lead levels. Second, in the case of pork, beans, and peas, there was

Table 5. Apparent lead contamination of Set 1 samples

No.	Food sample	Content in composite	Ottawa Set 1	Ottawa Set 2	Halifax Set 3	Toronto Set 4
15	Pork, baked[a]	—	273.3	5.3	15.8	< 3.0
15A	Pork, raw	100%	15.8	4.0	7.6	< 4.2
52	Potatoes, raw[a]	—	21.9	7.8	8.7	8.9
53	Potatoes, baked[a]	—	2547.0	40.6	15.7	3.5
54	Potatoes, boiled with skins[a]	—	11.5	4.0	18.3	3.0
58	Cabbage, cooked & coleslaw[a]	—	1137.0	4.6	10.1	20.0
61	Lettuce[a]	—	1025.0	2.3	7.9	18.2
64	Beans, raw & canned, cooked[a]	—	1689.0	72.6	45.7	38.0
64A	Beans, raw	50%	34.3	13.3	39.8	27.1
64B	Beans, canned	50%	735.7	120.2	140.5	38.0
65	Peas, raw & canned, cooked[a]	—	32.3	14.7	57.0	16.2
65A	Peas, raw	50%	13.2	9.6	11.0	7.7
65B	Peas, canned	50%	2371.0	23.9	58.6	12.8
69	Tomatoes, raw and cooked[a]	—	2165.0	2.5	3.5	—
69A	Tomatoes, cooked	50%	2547.0	1.4	—	8.4
69B	Tomatoes, raw	50%	914.1	2.0	2.8	< 3.2
73	Cucumber, raw and pickled[a]	—	228.2	8.3	7.4	< 4.6
73A	Cucumber, raw	50%	458.2	8.1	8.2	< 2.4

[a] Composite.

no correlation between the lead level in the composite and the level in the food item(s) that actually went into preparation of the composite.

The nature of the contamination source appeared to differ. The cucumber (No. 73A) and tomatoes (No. 69A) appeared to have been contaminated before preparation of the composites, whereas the canned peas (No. 65B) appeared to have been contaminated without processing after the composite was prepared. The beans (No. 64), pork (No. 15), and possibly baked potatoes (No. 53) appeared to have been contaminated during or after processing. The possibility also existed that all samples were contaminated after processing from their storage containers, but the approximate correlations between lead levels of the composites and their constituent foods suggest otherwise for cucumbers and tomatoes. Some of the steps taken to find the source of contamination are outlined below.

First, careful examination of all components of the utensils did not reveal any lead source, although one of the strainers that was not made of stainless steel was subsequently replaced by a stainless steel strainer. Some of the blenders used for the first part of the study were borrowed, and they could not be traced for evaluation afterward. Examination of the time the samples were processed revealed that all of the above fruit and vegetable samples were prepared within a 1-week period, soon after the installation of a new hot water tank and water supply line to the sample preparation laboratory. Belated but careful scrutiny of the hot and cold water supply showed that the water consistently contained less than 5 ng/mL lead, even after sitting in the pipes overnight. Contamination from the prewashed polyethylene sample bottles was unlikely because all the bottles for Set 1 were washed at the same time and randomly tested for contamination, yet most of the sample contamination was localized to a 1-week sample preparation period. Thus, all attempts to find the source of the contamination were unsuccessful.

The decision to analyze components of the composites as well as the composites themselves revealed that some of the canned foods (luncheon meats, fish, beans, citrus fruit, apple sauce, and cherries) contained appreciably higher lead levels than their fresh or frozen counterparts. In those cases in which lead-soldered cans were known to have been used (luncheon meat, fish, tomato juice, citrus fruit, apple sauce, pineapple, syrup, pudding, and pea, tomato, and meat soups) the lead level exceeded 100 ng/g in only the syrup, citrus fruit, tomato juice, and fish.

Pies with raisins contained higher lead levels (247 and 488 ng/g) than other pies, and raisins appeared to be a significant source of lead. Contamination of steak, ground beef, veal, lamb, poultry, and fish with lead during cooking was insignificant.

The mean, median, and range of lead concentrations by food category are given in Table 6. The higher mean levels for Set 1 vegetables and for Set 1 summary are probably unreliable because of the potential contamination discussed above.

The mean, median, and ranges of lead levels over all samples were 154, 21.6, and <0.2–2547 ng/g for Set 1, and 30, 14.7, and 1.4–407 ng/g for Set 2. The mean level of 30 ng/g for

Table 6. Lead levels in food categories

Category	Description	Set	*n*	Mean, ng/g	Median, ng/g	Range, ng/g
I	Milk and dairy products	1	13	7.98	1.7	0.28–48.6
		2	13	6.56	4.8	1.94–17.6
II	Meat and poultry	1	18	46.6	18.6	3.25–273
		2	18	18.5	12.4	3.03–67
III	Fish	1	6	89.8	10.8	7.70–298
		2	6	21.2	13.5	4.73–41.0
IV	Soups	1	4	23.8	20.2	10.4–39.3
		2	4	26.3	25.3	3.74–42.0
V	Bakery goods and cereals	1	28	48.4	23.1	3.49–488
		2	29	23.5	16.3	1.58–247
VI	Vegetables	1	36	494	50.1	3.31–2547
		2	37	29.3	12.0	1.42–182
VII	Fruit and fruit juices	1	27	55.2	19.8	< 0.6–356
		2	24	60.7	21.2	1.85–407
VIII	Fats and oils	1	3	11.5	9.9	< 0.2–24.5
		2	3	8.1	6.0	3.11–15.2
IX	Sugar and candies	1	8	67.2	51.6	9.3–177
		2	8	32.8	30.3	< 1.9–69.0
X	Beverages	1	5	11.7	7.1	2.71–28.5
		2	5	47.4	9.2	5.13–141
All composites		1 + 2	295	92.3	16.3	< 0.2–2547
		1	148	154.3	21.6	< 0.2–2547
		2	147	29.9	14.7	1.42–407

Set 2, which was not skewed by the suspected contamination source, agreed well with the mean level of 32 ng/g found in foods analyzed in a Canadian 24 h duplicate diet survey conducted in 1981 (3).

On the basis of lead concentrations found for Set 2, the estimated dietary ingestion of lead by all segments of the population was 36.4 μg/day, equivalent to 0.61 μg/kg body weight/day for a 60 kg adult. This was lower than the 54 μg/day reported in a Canadian 24 h duplicate diet study (3), and was well below the FAO/WHO provisional tolerable weekly intake of lead from all sources (7.1 μg/kg on a daily basis).

Total diet study structure.—As noted previously, the composites in Table 3 were changed slightly after the preliminary trials. The changes (1) were made to better reflect current food consumption patterns and to correlate the composites more closely with those used by the U.S. Food and Drug Administration. No changes were made to the composites on the basis of the trace element results; however, the practice of separately bottling specific foods that go into the preparation of some composites was continued. The precautions taken to avoid contamination during preparation and storage of the samples were continued for the main part of the study. For any new study, however, the lead results obtained in the first part of the study demonstrate the necessity of monitoring the water supply, carefully tracking all kitchen utensils and blenders, and pretesting all preparatory procedures for contamination by using a food low in metal concentration.

Additional quality control was desirable from an analytical viewpoint, and the decision was made to include a laboratory reference material in each analytical batch.

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DIETARY FIBER

Determination of Total, Soluble, and Insoluble Dietary Fiber in Foods—Enzymatic–Gravimetric Method, MES-TRIS Buffer: Collaborative Study

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A joint AOAC/AACC (American Association of Cereal Chemists) collaborative study of methods for the determination of soluble, insoluble, and total dietary fiber (SDF, IDF, and TDF) was conducted with 11 participating laboratories. The assay is based on a modification of the AOAC TDF method 985.29 and the SDF/IDF method collaboratively studied recently by AOAC. The principles of the method are the same as those for the AOAC dietary fiber methods 985.29 and 991.42, including the use of the same 3 enzymes (heat-stable α-amylase, protease, and amyloglucosidase) and similar enzyme incubation conditions. In the modification, minor changes have been made to reduce analysis time and to improve assay precision: (*1*) MES-TRIS buffer replaces phosphate buffer; (*2*) one pH adjustment step is eliminated; and (*3*) total volumes of reaction mixture and filtration are reduced. Eleven collaborators were sent 20 analytical samples (4 cereal and grain products, 3 fruits, and 3 vegetables) for duplicate blind analysis. The SDF, IDF, and TDF content of the foods tested ranged from 0.53 to 7.17, 0.59 to 60.53, and 1.12 to 67.56 g/100 g, respectively. The respective average RSD_R values for SDF, IDF, and TDF determinations by direct measurements were 13.1, 5.2, and 4.5%. The TDF values calculated by summing SDF and IDF were in excellent agreement with the TDF values measured independently. The modification did not alter the method performance with regard to mean dietary fiber values, yet it generated lower assay variability compared with the unmodified methods. The method for SDF, IDF, and TDF (by summing SDF and IDF) has been adopted first action by AOAC International.

Recent studies indicate that dietary fiber (DF) may be protective against cardiovascular diseases, diabetes, obesity, colon cancer, and other diverticular diseases (1–3). These findings have led to an increasing awareness of the importance of consuming foods rich in dietary fiber and the necessity of reliable methodology to determine DF content in foods and food products. Dietary fiber was initially defined as plant cell wall remnants that are resistant to hydrolysis by human alimentary enzymes (4). The definition has been extended to include all the polysaccharides and lignin in the diet that are resistant to the endogenous secretions of the human digestive tract (5). Accordingly, the term dietary fiber refers to nonstarch polysaccharides, resistant starch, and lignin; the AOAC total dietary fiber (TDF) method has evolved on this basis. This rapid enzymatic-gravimetric method was chosen by AOAC because it is simple and inexpensive for routine use in both quality control and research laboratories. The TDF method has passed several international interlaboratory studies (6–8) and was adopted final action by AOAC in 1986 (9), with a method change in 1988 (8). Subsequently, the AOAC TDF method, **985.29** (10), was adopted by government agencies in many countries, including the United States, Canada, Australia, Japan, Switzerland, Germany, and the Nordic countries.

The scope of the method was further expanded to give individual values for soluble and insoluble dietary fiber (SDF and IDF) (8, 11), which exhibit distinct physiological functions. Foods rich in water-insoluble dietary fiber are important in gas-

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trointestinal function, and foods rich in water-soluble dietary fiber have important metabolic effects on glucose and lipid metabolism (2). Separation of IDF from SDF by this method (8, 11) is based on water solubility of dietary fiber at atmospheric pressure, which has more physiological significance than do other SDF/IDF separation techniques that are based on the solubility of dietary fiber components either in dimethyl sulfoxide (DMSO) at elevated temperature (100°C) (H. Englyst, Dunn Clinical Research Laboratory, Cambridge, UK (1990), personal written communication) or in aqueous solutions under autoclave conditions (121°C, 15 psi) (12). In the 1988 and 1990 AOAC interlaboratory studies for TDF, SDF, and IDF determinations with the unmodified procedure, the precision of all 3 dietary fiber determinations was satisfactory, with the exception of values for SDF. Agreement of TDF values measured by independent analysis with those obtained by summation of SDF and IDF was excellent.

In the present collaborative study, the principles of the method are similar to those of method **985.29**, with the following minor modifications: (*1*) use of MES/TRIS buffer, pH 8.2 at 24°C, instead of phosphate buffer, pH 6.0; (*2*) elimination of a pH adjustment step for protease action; and (*3*) reduced volume of the reaction mixture and of the total filtration. These modifications were introduced to simplify the determination and improve assay efficiency while minimizing buffer precipitation. On the basis of the results of minicollaborative studies (13; G. Conti, Kraft General Foods, Tarrytown, NY, and D. Gordon, University of Missouri, Columbia, MO (1990), personal written communication) in the United States and of the methods comparison study in Mexico, this modification was adopted as official in Mexico in the spring of 1990. At that time, the National Institute of Nutrition in Mexico upgraded its fiber food tables and labeling from the previously accepted crude fiber to this new AOAC DF method, **991.43** (C. Rosado, National Institute of Nutrition, Mexico, Mexico City, Mexico (1990), personal written communication).

Various problematic food products, as well as analytically "normal" products that were identified from the previous studies (8, 11), were included in the present study to determine if the precision of dietary fiber determination (especially SDF) could be further improved with these modifications. The study was also designed to evaluate if any calculated dietary fiber values, such as TDF by summation of SDF and IDF, IDF by difference between TDF and SDF, and SDF by difference between TDF and IDF, could be successfully used as alternatives for the values determined by direct measurements. The present paper reports mean dietary fiber values and precision parameters on both an as-is basis and a dried basis (as analyzed) because reporting the values on an as-is basis is mandatory for nutrition labeling and because dietary fiber is often determined on dried foods.

Collaborative Study

The collaborators participating in this study were chemists in food industries, universities, commercial laboratories, and government laboratories in the United States and Canada. Each collaborator was sent the 3 enzymes (heat-stable α-amylase, protease, and amyloglucosidase), as well as Celite 545 AW, to be used in this study.

In the pretrial study, 4 test samples (oat bran and prunes in blind duplicate) were provided to collaborators. The oat bran and prunes were chosen for pretrial because they had been recognized as difficult to analyze in the previous AACC (14) and AOAC (11) studies. If the actual assay protocol in a laboratory was found to be similar to the suggested assay protocol, analysis of the main collaborative test samples could proceed. If not deemed acceptable, these laboratories were dropped from the study. Results from 2 collaborators who used a Tecator Fibertec apparatus and P-2 crucibles (pore size 40–90 μm), instead of regular vacuum source and coarse-pore crucibles (40–60 μm), were considered acceptable. Two other collaborators used a different grade of Celite, Analytical Filter Aid, instead of 545 AW, and their results were also considered acceptable in this study.

In the main collaborative study, 11 collaborators were sent 16 additional analytical samples in blind duplicate, which included the following 4 cereal and grain products, 3 fruits, and 3 vegetables: (a) barley, dehulled, rolled; (b) high-fiber cereal; (c) oat bran; (d) soy bran; (e) apricots; (f) prunes; (g) raisins; (h) carrots; (i) green beans; and (j) parsley. The barley, high-fiber cereal, and oat bran were purchased at a local supermarket. The soy bran and carrots were used in the AOAC 1989 collaborative study with the unmodified procedure. Results for these 2 foods showed good precision in the previous study. Thus, the foods were considered suitable for comparing the mean values generated by the 2 methods, because the comparison of means assumes homogeneity of variance for the 2 methods. All the fruit powders were obtained from VacuDry, Santa Rosa, CA; green beans and parsley were obtained from California Vegetable Concentrates, Modesto, CA.

Preparation of Analytical Samples

The barley, high-fiber cereal, and oat bran were ground in a Wiley mill with a 0.5 mm screen. If the fat content of any food had exceeded 10%, the food would have been defatted with petroleum ether (3 times with 25 mL/g food) before milling. [**Note**: Foods of unknown fat content should be defatted. High amounts of fat (>10%) in sample may interfere with DF determinations.] The fruit powders were desugared by using 10 volumes of 85% ethanol 2–3 times with decanting, then drying overnight at 40°C in an air oven with occasional turnover, and grinding in the Wiley mill (0.5 mm screen). The green beans and parsley were freeze-dried and ground by the supplier in a Glenn Hammermill with a 60-mesh screen.

Each analytical sample, except the prunes, was dried overnight in a 70°C vacuum oven and stored in vials containing desiccant until analyzed. The prunes contained 4.4% moisture and were analyzed as received. The loss of weight due to fat, sugar, or moisture removal was recorded to make appropriate corrections to the final percent DF. Each set was randomly split in half, and each subgroup was labeled with a separate number. Thus, 2 replicates of each of the foods were supplied blind to

each collaborator. Because none of the analytical samples contained more than 10% fat, fat extraction was not recommended.

Statistical Methods

Tests for outlying laboratories and outlying individual replicate values were performed according to methods described in the fourth (final) draft of AOAC "Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis" (15). The Cochran test was performed to remove data showing significantly greater variability among replicate (within-laboratory) analyses than did other laboratories for a given material. Grubbs tests were performed to remove laboratories with extreme averages.

Precision of the method was estimated by calculating the following parameters: standard deviations (SD); s_R, reproducibility SD (SD among laboratories including within laboratory); s_r, repeatability SD (SD within laboratory); and relative standard deviations (RSD = SD/$\bar{X}$ × 100) for reproducibility (RSD_R) and repeatability (RSD_r). Maximum tolerable differences (SD × 2.8) were also calculated for reproducibility (R = s_R × 2.8) and repeatability (r = s_r × 2.8).

To transform the dietary fiber content and its standard deviation from a dried basis to a fresh weight basis, the following formula was used (16):

% Dietary fiber (or SD) fresh weight basis = % DF (or SD) in dried product × (100 – % moisture fresh weight basis)/(100 – % moisture in dried product), i.e., % DF (or SD) fresh weight basis = % DF (or SD) in dried product × conversion factor.

The conversion factors that were calculated for each product are as follows (the conversion factors for fruits included the factors for both dewatering and desugaring steps): barley, 0.935000; high-fiber cereal, 0.973000; oat bran, 0.927400; soy bran, 0.935000; apricots, 0.016841; prunes, 0.308000; raisins, 0.066130; carrots, 0.089000; green beans, 0.096100; and parsley, 0.092084.

991.43 Total, Soluble, and Insoluble Dietary Fiber in Foods Enzymatic-Gravimetric Method, MES-TRIS Buffer

First Action 1991

(Applicable to processed foods, grain and cereal products, fruits, and vegetables.)

Method Performance:

See Table **991.43A** for method performance data.

A. Principle

Duplicate samples of dried foods, fat-extracted if containing >10% fat, undergo sequential enzymatic digestion by heat stable α-amylase, protease, and amyloglycosidase to remove starch and protein. For total dietary fiber (TDF), enzyme digestate is treated with alcohol to precipitate soluble dietary fiber before filtering, and TDF residue is washed with alcohol and acetone, dried, and weighed. For insoluble and soluble dietary fiber (IDF and SDF), enzyme digestate is filtered, and residue (IDF) is washed with warm water, dried, and weighed. For SDF, combined filtrate and washes are precipitated with alcohol, filtered, dried, and weighed. TDF, IDF, and SDF residue values are corrected for protein, ash, and blank.

B. Apparatus

(a) *Beakers.*—400 or 600 mL tall form.

(b) *Filtering crucible.*—With fritted disk, coarse, ASTM 40–60 μm pore size, Pyrex 60 mL (Corning No. 36060 Buchner, or equivalent). Prepare as follows. Ash overnight at 525° in muffle furnace. Let furnace temperature fall below 130° before removing crucibles. Soak crucibles 1 h in 2% cleaning solution at room temperature. Rinse crucibles with H_2O and then deionized H_2O; for final rinse, use 15 mL acetone and then air-dry. Add ca 1.0 g Celite to dry crucibles, and dry at 130° to constant weight. Cool crucible ca 1 h in desiccator, and record weight, to nearest 0.1 mg, of crucible plus Celite.

(c) *Vacuum system.*—Vacuum pump or aspirator with regulating device. Heavy walled filtering flask, 1 L, with side arm. Rubber ring adaptors, for use with filtering flasks.

(d) *Shaking water baths.*—(*1*) Capable of maintaining 98 ±2°, with automatic on-and-off timer. (*2*) Constant temperature, adjustable to 60°.

(e) *Balance.*—Analytical, sensitivity ±0.1 mg.

(f) *Muffle furnace.*—Capable of maintaining 525 ±5°.

(g) *Oven.*—Capable of maintaining 105 and 130 ±3°.

(h) *Desiccator.*—With SiO_2 or equivalent desiccant. Biweekly, dry desiccant overnight at 130°.

(i) *pH meter.*—Temperature compensated, standardized with pH 4.0, 7.0, and 10.0 buffer solutions.

(j) *Pipetters.*—With disposable tips, 50–300 μL and 5 mL capacity.

(k) *Dispensers.*—Capable of dispensing 15 ±0.5 mL for 78% EtOH, 95% EtOH, and acetone; 40 ±0.5 mL for buffer.

(l) *Magnetic stirrers and stir bars.*

C. Reagents

Use deionized water throughout.

(a) *Ethanol solutions.*—(*1*) 85%. Place 895 mL 95% ethanol into 1 L volumetric flask, dilute to volume with H_2O. (*2*) 78%. Place 821 mL 95% ethanol into 1 L volumetric flask, dilute to volume with H_2O.

(b) *Heat-stable α-amylase solution.*—Cat. No. A 3306, Sigma Chemical Co., St. Louis, MO 63178, or Termamyl 300L, Cat. No. 361-6282, Novo-Nordisk, Bagsvaerd, Denmark, or equivalent. Store at 0–5°C.

(c) *Protease.*—Cat. No. P 3910, Sigma Chemical Co., or equivalent. Prepare 50 mg/mL enzyme solution in MES/TRIS buffer fresh daily. Store at 0–5°C.

(d) *Amyloglucosidase solution.*—Cat. No. AMG A9913, Sigma Chemical Co., or equivalent. Store at 0–5°.

(e) *Diatomaceous earth.*—Acid washed (Celite 545 AW, No. C8656, Sigma Chemical Co., or equivalent).

(f) *Cleaning solution.*—Liquid surfactant-type laboratory cleaner, designed for critical cleaning (Micro®, International

Table 991.43A. Method performance for 991.43, dietary fiber in foods

Food	Mean, g/100 g	s_r	s_R	RSD_r, %	RSD_R, %
Total dietary fiber (TDF)					
Barley	12.25	0.36	0.85	2.88	6.89
High fiber cereal	33.73	0.70	0.94	2.08	2.79
Oat bran	16.92	1.06	2.06	6.26	12.17
Soy bran	67.14	1.01	1.06	1.50	1.58
Apricots	1.12	0.01	0.01	0.89	0.89
Prunes	9.29	0.13	0.40	1.40	4.31
Raisins	3.13	0.09	0.15	2.88	4.79
Carrots	3.93	0.13	0.13	3.31	3.31
Green beans	2.89	0.07	0.07	2.42	2.42
Parsley	2.66	0.07	0.14	2.63	5.26
Soluble dietary fiber (SDF)					
Barley	5.02	0.40	0.62	8.01	12.29
High fiber cereal	2.78	0.44	0.56	15.83	20.14
Oat bran	7.17	0.72	1.14	10.04	15.90
Soy bran	6.90	0.30	0.60	4.35	8.70
Apricots	0.53	0.02	0.02	3.77	3.77
Prunes	5.07	0.11	0.31	2.17	6.11
Raisins	0.73	0.05	0.16	6.85	21.92
Carrots	1.10	0.07	0.18	6.36	16.36
Green beans	1.02	0.08	0.11	7.84	10.78
Parsley	0.64	0.03	0.10	4.69	15.63
Insoluble dietary fiber (IDF)					
Barley	7.05	0.61	0.61	8.62	8.62
High fiber cereal	30.52	0.44	0.71	1.44	2.33
Oat bran	9.73	0.85	1.17	8.74	12.02
Soy bran	60.53	0.70	0.70	1.16	1.16
Apricots	0.59	0.02	0.02	3.39	3.39
Prunes	4.17	0.07	0.09	1.68	2.16
Raisins	2.37	0.04	0.07	1.69	2.95
Carrots	2.81	0.09	0.16	3.20	5.69
Green beans	2.01	0.08	0.08	3.98	3.98
Parsley	2.37	0.12	0.24	5.06	10.13
Total dietary fiber (SDF + IDF)					
Barley	12.14	0.39	0.70	3.21	5.77
High fiber cereal	33.30	0.63	0.90	1.89	2.70
Oat bran	16.90	0.99	1.49	5.86	8.82
Soy bran	67.56	0.56	0.94	0.83	1.39
Apricots	1.12	0.02	0.02	1.79	1.79
Prunes	9.37	0.12	0.30	1.28	3.20
Raisins	3.10	0.05	0.18	1.61	5.81
Carrots	3.92	0.11	0.13	2.81	3.32
Green beans	3.03	0.09	0.12	2.97	3.96
Parsley	3.01	0.12	0.23	3.99	7.64

Products Corp., Trenton, NJ 08016, or equivalent). Prepare 2% solution in H_2O.

(g) *MES.*—2-(*N*-Morpholino)ethanesulfonic acid (No. M-8250, Sigma Chemical Co., or equivalent.)

(h) *TRIS.*—Tris(hydroxymethyl)aminomethane (No. T-1503, Sigma Chemical Co., or equivalent).

(i) *MES/TRIS buffer solution.*—0.05M MES, 0.05M TRIS, pH 8.2 at 24°. Dissolve 19.52 g MES and 12.2 g TRIS in 1.7 L H_2O. Adjust pH to 8.2 with 6N NaOH, and dilute to 2 L with H_2O. (Note: It is important to adjust pH to 8.2 at 24°. However, if buffer temperature is 20°, adjust pH to 8.3; if temperature is 28°, adjust pH to 8.1. For deviations between 20 and 28°, adjust by interpolation.)

(j) *Hydrochloric acid solution.*—0.561N. Add 93.5 mL 6N HCl to ca 700 mL H_2O in 1 L volumetric flask. Dilute to 1 L with H_2O.

D. Enzyme Purity

To ensure absence of undesirable enzymatic activities and presence of desirable enzymatic activities, run standards listed in Table **991.43B** each time enzyme lot changes or at maximum interval of 6 months.

E. Sample Preparation and Digestion

Prepare samples as in **985.29E** (if fat content of sample is unknown, defat before determining dietary fiber). For high sugar samples, desugar before determining dietary fiber by extracting 2–3 times with 85% EtOH, 10 mL/g, decanting, and then drying overnight at 40°.

Run 2 blanks/assay with samples to measure any contribution from reagents to residue.

Weigh duplicate 1.000 ±0.005 g samples (M_1 and M_2), accurate to 0.1 mg, into 400 mL (or 600 mL) tall form beakers. Add 40 mL MES/TRIS buffer solution, pH 8.2, to each. Stir on magnetic stirrer until sample is completely dispersed (to prevent lump formation, which would make test material inaccessible to enzymes). Add 50 µL heat-stable α-amylase solution, stirring at low speed. Cover beakers with Al foil, and incubate in 95–100° H_2O bath 15 min with continuous agitation. Start timing once bath temperature reaches 95° (total of 35 min is normally sufficient).

Remove all beakers from bath, and cool to 60°. Remove foil. Scrape any ring from inside of beaker and disperse any gels in bottom of beaker with spatula. Rinse beaker walls and spatula with 10 mL H_2O.

Add 100 µL protease solution to each beaker. Cover with Al foil, and incubate 30 min at 60 ±1° with continuous agitation. Start timing when bath temperature reaches 60°.

Remove foil. Dispense 5 mL 0.561N HCl into beakers while stirring. Adjust pH to 4.0–4.7 at 60°, by adding 1N NaOH solution or 1N HCl solution. (Note: It is important to check and adjust pH while solutions are 60° because pH will increase at lower temperatures.) (Most cereal, grain, and vegetable products do not require pH adjustment. Once verified for each laboratory, pH checking procedure can be omitted. As a precaution, check pH of blank routinely; if outside desirable range, check samples also.)

Add 300 µL amyloglucosidase solution while stirring. Cover with Al foil, and incubate 30 min at 60° ±1° with constant agitation. Start timing once bath reaches 60°.

F. Determination of Total Dietary Fiber

To each digested sample, add 225 mL (measured after heating) 95% EtOH at 60°. Ratio of EtOH to sample volume should be 4:1. Remove from bath, and cover beakers with large sheets of Al foil. Let precipitate form 1 h at room temperature.

Wet and redistribute Celite bed in previously tared crucible *B*(b), using 15 mL 78% EtOH from wash bottle. Apply suction to crucible to draw Celite onto fritted glass as even mat.

Filter alcohol-treated enzyme digestate through crucible. Using wash bottle with 78% EtOH and rubber spatula, quantitatively transfer all remaining particles to crucible. (Note: If some samples form a gum, trapping the liquid, break film with spatula.)

Using vacuum, wash residue 2 times each with 15 mL portions of 78% EtOH, 95% EtOH, and acetone. Dry crucible containing residue overnight in 105° oven. Cool crucible in desiccator ca 1 h. Weigh crucible, containing dietary fiber residue and Celite, to nearest 0.1 mg, and calculate residue weight by subtracting weight of dry crucible with Celite, *B*(b).

Use one duplicate from each sample to determine protein, by method **960.52**, using N × 6.25 as conversion factor. For ash analysis, incinerate second duplicate 5 h at 525°. Cool in desiccator, and weigh to nearest 0.1 mg. Subtract weight of crucible and Celite, *B*(b), to determine ash wt.

G. Determination of Insoluble Dietary Fiber

Wet and redistribute Celite bed in previously tared crucible, *B*(b), using ca 3 mL H_2O. Apply suction to crucible to draw Celite into even mat.

Table 991.43B. Enzyme purity

Standard	Activity tested	Wt of Std, g	Expected rec., %
Citrus pectin	Pectinase	0.1–0.2	95–100
Arabinogalactan	Hemicellulase	0.1–0.2	95–100
β-Glucan	β-Glucanase	0.1–0.2	95–100
Wheat starch	α-Amylase + AMG	1.0	0–1
Corn starch	α-Amylase + AMG	1.0	0–1
Casein	Protease	0.3	0–1

Filter enzyme digestate, from *E*, through crucible into filtration flask. Rinse beaker, and then wash residue 2 times with 10 mL 70° H_2O. Combine filtrate and water washings, transfer to pretared 600 mL tall form beaker, and reserve for determination of soluble dietary fiber, *H*.

Using vacuum, wash residue 2 times each with 15 mL portions of 78% EtOH, 95% EtOH, and acetone. (Note: Delay in washing IDF residues with 78% EtOH, 95% EtOH, and acetone may cause inflated IDF values.)

Use duplicates to determine protein and ash as in *F*.

H. Determination of Soluble Dietary Fiber

Proceed as for insoluble dietary fiber determination through instruction to combine the filtrate and water washings in pretared 600 mL tall form beakers. Weigh beakers with combined solution of filtrate and water washings, and estimate volumes.

Add 4 volumes of 95% EtOH preheated to 60°. Use portion of 60° EtOH to rinse filtering flask from IDF determination. Alternatively, adjust weight of combined solution of filtrate and water washings to 80 g by addition of H_2O, and add 320 mL 60° 95% EtOH. Let precipitate form at room temperature 1 h.

Follow TDF determination, *F*, from "Wet and redistribute Celite bed ...."

I. Calculations

Blank (*B*, mg) determination:

$$B = [(BR_1 + BR_2)/2] - P_B - A_B$$

where BR_1 and BR_2 = residue weights (mg) for duplicate blank determinations; and P_B and A_B = weights (mg) of protein and ash, respectively, determined on first and second blank residues.

Dietary fiber (*DF*, g/100 g) determination:

$$DF = \{[(R_1 + R_2)/2] - P - A - B\}/[(M_1 + M_2)/2] \times 100$$

where R_1 and R_2 = residue weights (mg) for duplicate samples; *P* and *A* = weights (mg) of protein and ash, respectively, determined on first and second residues; *B* = blank weight (mg); and M_1 and M_2 = weights (mg) for samples.

Total dietary fiber determination: Determine either by independent analysis, as in *F*, or by summing IDF and SDF, as in *G* and *H*.

Ref.: JAOAC **75**, May/June issue (1992)

Results and Discussion

Recent surveys showed a mean total dietary fiber intake of 11.2–22.2 g/day in the population of various countries, including the United States (17), Japan (18), the United Kingdom (19, 20), and Sweden (21). These intakes are less than the recommended dietary goals of 25–35 g TDF/day (2). People who consume more than 20 g TDF/day had 3 or more servings of fruits and vegetables in addition to whole grain cereals in the diet (17). Approximately 40–50% DF intake is from fruits and vegetables and 30–50% from cereals and grains in those populations (18, 20–22). Although fruits and vegetables contain small amounts of DF on an as-is basis, they are consumed in sufficiently large quantities to make an appreciable contribution to dietary fiber intake (14). Fruits and vegetables, as well as cereal and grain products, are also good sources of SDF (8, 11). Thus, it is important to investigate the performance of the method used to determine soluble, insoluble, and total dietary fiber on low-fiber products such as fruits and vegetables, as well as on grains and cereals. Accordingly, this study included 4 cereal and grain products, 3 fruits, and 3 vegetables to cover a variety of foods at a wide range of dietary fiber concentrations. In the future, nutrition labeling may require reporting on an as-is basis, even though dietary fiber is usually determined in the dried food. Consequently, mean dietary fiber values and precision parameters were reported on a dried basis as well as an as-is basis. With most dry-type products such as grains and cereals, changing the basis made little difference in the magnitude of fiber content and conclusions regarding precision parameters such as s_r and s_R. However, the dietary fiber content and standard deviations for fruits and vegetables differed by 1–2 orders of magnitude. In all cases, the relative standard deviations, RSD_r and RSD_R, were not affected by changing the basis.

In the present study, a modification of the AOAC DF method **985.29** was used to determine SDF, IDF, and TDF. The method was modified, as described earlier, to improve analytical productivity. Preliminary results indicated that this modification improved assay precision. This study investigated whether the modification could improve the method performance for commodities such as prunes, raisins, apricots, parsley, and oat bran. In the previous AOAC/AACC collaborative studies (11, 14), these commodities were difficult to analyze in the evaluation of the precision of the method in the worst cases. The present study also included foods such as soy bran and carrots, for which the DF determination was found to be precise.

Results reported by each participating laboratory are presented in Table 1, where blind duplicate results have been paired for each food material. The tested foods covered a wide range of dietary fiber concentrations (Tables 1–5). Total dietary fiber content (g/100 g) on an as-is basis ranged from 1.12 for apricots to 67.14 for soy bran (13.10–71.80 on dry weight basis). Soluble dietary fiber content (g/100 g) ranged from 0.53 for apricot to 7.17 for oat bran (2.86–31.42 on a dried basis). Insoluble dietary fiber content (g/100 g) ranged from 0.59 for apricots to 60.53 for soy bran (7.54–64.74 on a dried basis).

The AOAC statistical parameters for both repeatability and reproducibility for each food are summarized in Tables 1–5 for soluble, insoluble, and total dietary fiber determination. All the dietary fiber determinations by direct measurement showed excellent precision. For TDF determination, the RSD_R ranged from 0.89% for apricots to 12.17% for oat bran, with an average of 4.44%. The s_R of TDF values (g/100 g) was in the range of 0.01 for apricots and 2.06 for oat bran on an as-is basis and 0.77 for apricots/green beans and 2.38 for raisins on a dried basis. The RSD_R of SDF determinations ranged from 3.77% for apricots to 21.92% for raisins; the mean RSD_R for all 10 prod-

Table 1. Collaborative results of dietary fiber determination in blind duplicates by the AOAC DF method 991.43[a]

Laboratory	Direct measurement			Calculation		
	TDF	SDF	IDF	C-TDF (SDF + IDF)	C-SDF (TDF - IDF)	C-IDF (TDF - SDF)
			Barley, g DF/100 g			
1	12.74	4.88	7.88	12.76	4.86	7.86
	12.69	5.58	7.14	12.72	5.55	7.11
2	12.96	5.14	7.47	12.61	5.49	7.82
	12.64	5.41	7.46	12.87	5.18	7.23
3	12.45	6.98	6.82	13.80	5.63	5.47
	12.29	5.92	8.44	14.36	3.85	6.37
4	13.32	4.96	8.49[b]	13.45	4.83	8.36
	12.97	5.07	8.09[b]	13.16	4.88	7.90
5	12.05	5.92	8.58	14.50[c]	3.47	6.13
	11.82	4.74	6.55	11.29[c]	5.27	7.08
6	14.49	6.46	7.38	13.84	7.11	8.03
	14.82	6.06	7.82	13.88	7.00	8.76
7	12.98	4.82	7.52	12.34	5.46	8.16
	13.64	4.31	7.08	11.39	6.56	9.33
8	12.14	4.94	7.29	12.23	4.83	7.20
	12.29	5.04	7.56	12.60	4.73	7.25
9	—	4.95	7.54	12.49	—	—
	NA[d]	5.21	7.59	12.80	—	—
10	14.43	5.88	7.97	13.85	6.46	8.55
	13.28	5.91	7.35	13.26	5.93	7.37
11	14.38	5.33	7.92	13.25	6.46	9.05
	13.57	4.63	7.36	11.99	6.21	8.94
$\bar{X}$	13.10	5.37	7.54	12.98	5.49	7.70
s_r	0.38	0.43	0.65	0.42	0.65	0.55
s_R	0.91	0.66	0.65	0.75	0.98	1.03
RSD_r, %	2.90	8.01	8.62	3.24	11.85	7.14
RSD_R, %	6.95	12.29	8.62	5.78	17.97	13.38
			High-fiber cereal, g DF/100 g			
1	33.58	3.27	31.37	34.64	2.21	30.31
	34.88	3.23	30.68	33.91	4.20	31.65
2	34.25	1.96	31.60	33.56	2.65	32.29
	33.35	2.03	31.80	33.83	1.55	31.32
3	34.37	3.43	32.55	35.98	1.82	30.94
	33.14	1.96	33.15	35.11	-0.01	31.18
4	34.39	2.48	31.21	33.69	3.18	31.91
	34.87	2.42	31.05	33.47	3.82	32.45
5	34.93	3.65	31.74	35.39	3.19	31.28
	34.84	3.53	31.31	34.75	3.44	31.22
6	36.94	3.47	30.84	34.31	6.10	33.47
	35.51	3.38	30.95	34.33	4.56	32.13
7	35.96	3.29	32.26	35.55	3.70	32.67
	35.73	2.57	31.52	34.09	4.21	33.16
8	34.75	2.70	30.47	33.17	4.28	32.05
	33.19	3.26	31.52	34.78	1.67	29.93
9	NA[d]	2.53	30.04	32.57	—	—
	—	2.23	30.89	33.12	—	—
10	35.34	3.71	31.81	35.52	3.53	31.63
	34.09	2.54	31.61	34.15	2.48	31.59

Table 1. ***Continued***

Laboratory	Direct measurement			Calculation		
	TDF	SDF	IDF	C-TDF (SDF + IDF)	C-SDF (TDF − IDF)	C-IDF (TDF − SDF)
11	34.64	2.67	30.40	33.07	4.24	31.97
	34.70	2.54	31.35	33.89	3.35	32.16
$\bar{X}$	34.67	2.86	31.37	34.22	3.21	31.76
s_r	0.72	0.45	0.45	0.65	1.01	0.70
s_R	0.97	0.58	0.73	0.93	1.36	0.87
RSD_r, %	2.08	15.73	1.43	1.90	31.46	2.20
RSD_R, %	2.80	20.28	2.33	2.72	42.37	2.74
	Oat bran (pretrial sample), g DF/100 g					
1	19.95	7.72	12.53	20.25	7.42	12.23
	21.11	8.44	12.16	20.60	8.95	12.67
2	17.25	8.98	9.35	18.33	7.90	8.29
	18.46	8.82	9.72	18.54	8.74	9.64
3	17.24	10.39	8.76	19.15	8.48	6.85
	16.05	7.79	9.44	17.23	6.61	8.26
4	19.54	6.85	9.47	16.32	10.07	12.69
	20.53	7.03	10.55	17.58	9.98	13.50
5	12.51	4.68	11.79	16.47	0.72	7.83
	15.30	6.77	10.11	16.89	5.19	8.53
6	17.52	8.15	10.11	18.34	7.33	9.37
	17.54	7.70	9.89	17.59	7.65	9.84
7	21.70	5.20	10.20	15.40	11.50	16.50
	20.60	6.20	10.70	16.90	9.90	14.40
8	19.09	8.17	12.52	20.69	6.57	10.92
	16.07	7.78	9.66	17.44	6.41	8.29
	17.86	8.65	11.23	19.88	6.63	9.21
	18.04	8.65	11.98	20.63	6.06	9.39
9	16.85	8.12	9.06	17.18	7.79	8.73
	16.63	7.80	8.73	16.53	7.90	8.83
10	18.99	8.37	9.47	17.84	9.52	10.62
	18.73	7.21	11.21	18.42	7.52	11.52
11	21.81	7.79	10.55	18.34	11.26	14.02
	18.60	8.30	12.64	20.94	5.96	10.30
$\bar{X}$	18.25	7.73	10.50	18.23	7.75	10.52
s_r	1.14	0.78	0.92	1.07	1.54	1.10
s_R	2.22	1.23	1.26	1.61	2.28	2.50
RSD_r, %	6.25	10.09	8.76	5.87	19.87	10.46
RSD_R, %	12.16	15.91	12.00	8.83	29.42	23.76
	Soy bran, g DF/100 g					
1	71.02	7.60	64.70	72.30	6.32	63.42
	71.13	7.78	64.06	71.84	7.37	63.65
2	71.68	7.02	65.63	72.65	6.05	64.66
	70.41	6.61	64.81	71.42	5.60	63.80
3	70.88	7.81	64.16	71.97	6.72	63.07
	71.27	6.85	65.64	72.49	5.63	64.42
4	72.96	7.79	64.73	72.52	8.23	65.17
	70.38	7.61	64.59	72.20	5.79	62.77
5	71.39	7.58	64.92	72.50	6.47	63.81
	71.82	7.96	64.68	72.64	7.14	63.86

Table 1. ***Continued***

Laboratory	Direct measurement			Calculation		
	TDF	SDF	IDF	C-TDF (SDF + IDF)	C-SDF (TDF - IDF)	C-IDF (TDF - SDF)
6	74.61	7.30	65.31	72.61	9.30	67.31
	71.11	7.87	64.78	72.65	6.33	63.24
7	73.23	7.62	65.10	71.72	8.13	65.61
	73.89	7.32	63.97	71.29	9.92	66.57
8	72.20	7.04	67.01[b]	74.05	5.19	65.16
	72.41	6.97	66.31[b]	73.28	6.10	65.44
9	NA[d]	6.04	64.37	70.41	—	—
	—	6.50	64.07	70.57	—	—
10	71.97	8.82	63.93	72.75	8.04	63.15
	71.30	8.28	66.18	74.46	5.12	63.02
11	70.57	6.86	64.04	70.90	6.53	63.71
	71.71	7.18	65.16	72.34	6.55	64.53
$\bar{X}$	71.80	7.38	64.74	72.25	6.83	64.32
s_r	1.08	0.32	0.75	0.60	1.23	1.15
s_R	1.14	0.64	0.75	1.00	1.32	1.24
RSD_r, %	1.50	4.34	1.16	0.83	18.01	1.79
RSD_R, %	1.59	8.67	1.16	1.38	19.33	1.93
			Apricots, g DF/100 g			
1	67.78	31.04	34.55	65.59	33.23	36.74
	67.10	31.38	36.86	68.24	30.24	35.72
2	66.51	32.14	35.06	67.20	31.45	34.37
	67.38	30.65	36.60	67.25	30.78	36.73
3	66.36	31.59	36.72	68.31	29.64	34.77
	67.96	33.33	35.62	68.95	32.34	34.63
4	65.63	31.83	34.43	66.26	31.20	33.80
	66.16	31.25	34.82	66.07	31.34	34.91
5	67.68	31.44	35.45	66.89	32.23	36.24
	67.15	30.99	34.64	65.63	32.51	36.16
6	66.47	32.21	33.79	66.00	32.68	34.26
	66.09	31.42	35.47	66.89	30.62	34.67
7	71.24[b]	30.36	36.05	66.41	35.19	40.88
	70.19[b]	31.26	35.06	66.30	34.53	38.93
8	67.38	32.63	35.00	67.63	32.38	34.75
	67.81	30.08	36.14	66.22	31.67	37.73
9	NA[d]	27.45[b]	35.76	63.21[b]	—	—
	—	29.05[b]	35.71	64.76[b]	—	—
10	66.82	31.65	35.44	67.09	31.38	35.17
	65.66	30.40	33.52	63.92	32.14	35.26
11	65.91	27.24[b]	33.99	61.23[b]	31.92	38.67
	66.56	23.26[b]	33.18	56.44[b]	33.38	43.30
$\bar{X}$	66.80	31.42	35.20	66.75	31.73	36.38
s_r	0.60	0.92	0.91	1.11	1.16	1.45
s_R	0.77	0.93	1.02	1.17	1.16	2.49
RSD_r, %	0.90	2.93	2.59	1.66	3.66	3.99
RSD_R, %	1.15	2.96	2.90	1.75	3.66	6.84
			Prunes (pretrial sample), g DF/100 g			
1	29.59	16.92	13.50	30.42	16.09	12.67
	30.36	17.33	13.59	30.92	16.77	13.03

Table 1. ***Continued***

Laboratory	Direct measurement			Calculation		
	TDF	SDF	IDF	C-TDF (SDF + IDF)	C-SDF (TDF – IDF)	C-IDF (TDF – SDF)
2	28.84	15.92	13.58	29.50	15.26	12.92
	29.27	16.78	13.05	29.83	16.22	12.49
3	32.14	18.33	13.28	31.61	18.86	13.81
	32.07	17.94	13.67	31.61	18.40	14.13
4	29.85	16.04	14.24	30.28	15.61	13.81
	30.82	15.83	13.63	29.46	17.19	14.99
5	28.15	16.20	13.28	29.48	14.87	11.95
	28.11	17.09	13.41	30.50	14.70	11.02
6	30.54	17.17	13.78	30.95	16.76	13.37
	30.95	17.74	13.85	31.59	17.10	13.21
7	32.50	14.33	15.51[c]	29.84	16.99	18.17
	31.70	14.70	14.33[c]	29.03	17.37	17.00
8	30.94	16.10	15.63[b]	31.73	15.31	14.84
	30.45	16.24	15.46[b]	31.70	14.99	14.21
	30.63	16.88	15.07[b]	31.95	15.66	13.75
	29.82	16.01	15.35[b]	31.36	14.47	13.81
9	29.04	15.97	13.46	29.43	15.58	13.07
	28.62	15.95	13.62	29.57	15.00	12.67
10	30.91[c]	17.67	13.16	30.83	17.75	13.24
	28.11[c]	17.21	13.21	30.42	14.90	10.90
11	29.60	15.52	13.62	29.14	15.98	14.08
	29.47	15.56	13.77	29.33	15.70	13.91
$\bar{X}$	30.16	16.48	13.54	30.43	16.14	13.63
s_r	0.41	0.37	0.22	0.38	0.74	0.66
s_R	1.30	1.02	0.28	0.98	1.23	1.63
RSD_r, %	1.36	2.25	1.62	1.25	4.58	4.84
RSD_R, %	4.31	6.19	2.07	3.22	7.62	11.96
	Raisins, g DF/100 g					
1	44.99	9.45	36.02	45.47	8.97	35.54
	44.23	10.06	36.39	46.45	7.84	34.17
2	45.27	—	35.60	—	9.67	—
	43.73	8.03	35.84	43.90	7.89	35.70
3	48.83	14.23	36.47	50.70	12.36	34.60
	49.35	12.08	37.19	49.27	12.16	37.27
4	46.36	10.64	35.41	46.05	10.95	35.72
	45.67	9.29	35.63	44.92	10.04	36.38
5	47.06	13.87	36.30	50.17	10.76	33.19
	47.27	14.74	35.69	50.43	11.58	32.53
6	50.73	11.85	36.64	48.49	14.09	38.88
	49.04	11.26	35.94	47.20	13.10	37.78
7	50.84	8.60	36.30	44.90	14.54	42.24
	49.33	8.38	37.94	46.32	11.39	40.95
8	51.62	11.65	42.48[c]	54.13[c]	9.14	39.97
	46.50	10.76	37.74[c]	48.50[c]	8.76	35.74
9	NA[d]	11.88	36.49	48.37	—	—
	—	11.45	36.13	47.58	—	—
10	48.82	14.36	33.36	47.72	15.46	34.46
	46.72	13.65	33.43	48.08	12.29	33.07
11	44.98	7.79	34.62	42.41	10.36	37.19
	45.53	6.86	34.20	41.06	11.33	38.67

Table 1. ***Continued***

Laboratory	Direct measurement			Calculation		
	TDF	SDF	IDF	C-TDF (SDF + IDF)	C-SDF (TDF - IDF)	C-IDF (TDF - SDF)
$\bar{X}$	47.34	10.99	35.83	46.81	11.13	36.53
s_r	1.41	0.72	0.54	0.77	1.18	1.39
s_R	2.38	2.39	1.08	2.70	2.18	2.76
RSD_r, %	2.98	6.55	1.51	1.64	10.60	3.81
RSD_R, %	5.03	21.75	3.01	5.77	19.59	7.56
			Carrots, g DF/100 g			
1	43.22	11.35	32.18	43.53	11.04	31.87
	45.50	11.48	33.18	44.66	12.32	34.02
2	43.28	10.03	32.96	42.99	10.32	33.25
	42.17	9.90	32.86	42.76	9.31	32.27
3	45.38	15.16	29.32	44.48	15.96	30.12
	42.16	14.28	31.42	45.70	10.74	27.88
4	44.01	12.78	30.84	43.62	13.17	31.23
	43.56	11.45	31.66	43.11	11.90	32.11
5	43.51	12.16	34.69	46.85	8.82	31.35
	46.19	12.83	31.83	44.66	14.36	33.36
6	45.10	12.68	30.68	43.36	14.42	32.42
	42.49	14.05	30.07	44.12	12.42	28.44
7	44.66	10.85	32.05	42.90	12.61	33.81
	46.14	11.54	32.30	43.84	13.84	34.60
8	43.79	10.47	32.39	42.86	11.40	33.32
	45.39	12.97	34.39	47.36	11.00	32.42
10	45.01	16.25	28.31	44.62	16.64	28.76
	44.61	16.61	27.89	44.50	16.72	28.00
11	44.76	10.32	32.27	42.59	12.49	34.44
	42.49	10.81	30.89	41.70	11.60	31.68
$\bar{X}$	44.17	12.40	31.61	44.01	12.55	31.77
s_r	1.42	0.77	1.02	1.23	1.85	1.43
s_R	1.42	2.04	1.79	1.42	2.25	2.15
RSD_r, %	3.21	6.21	3.23	2.79	14.74	4.50
RSD_R, %	3.21	16.45	5.66	3.23	17.93	6.77
			Green beans, g DF/100 g			
1	29.75	11.52	20.54	32.06	9.21	18.23
	30.33	11.32	21.16	32.48	9.17	19.01
2	30.56	10.48	20.47	30.95	10.09	20.08
	30.00	10.44	20.60	31.04	9.40	19.56
3	30.61	12.20	20.72	32.92	9.89	18.41
	29.44	11.25	21.08	32.33	8.36	18.19
4	30.22	11.13	20.07	31.20	10.15	19.09
	29.92	8.82	22.19	31.01	7.33	20.70
5	29.13	10.96	22.04	33.00	7.09	18.17
	30.79	10.96	—	—	—	19.83
6	30.95	10.63	21.70	32.33	9.23	20.30
	30.88	10.37	21.41	31.78	9.47	20.51
7	32.18	9.09	22.81	31.90	9.37	23.09
	31.61	11.08	20.68	31.76	10.93	20.53
8	30.89	12.06	20.83	32.89	10.06	18.83
	29.01	10.79	20.44	31.23	8.57	18.22

Table 1. ***Continued***

Laboratory	Direct measurement			Calculation		
	TDF	SDF	IDF	C-TDF (SDF + IDF)	C-SDF (TDF – IDF)	C-IDF (TDF – SDF)
9	NA[d]	9.21	21.40	30.60	—	—
	—	8.95	20.66	29.61	—	—
10	30.64	11.55	20.39	31.94	10.25	19.09
	29.93	12.00	20.12	32.12	9.81	17.93
11	30.23	9.63	21.33	30.96	8.90	20.60
	30.62	8.52	19.06	27.58	11.56	22.10
$\bar{X}$	30.10	10.59	20.94	31.51	9.41	19.62
s_r	0.77	0.78	0.88	0.90	1.12	0.92
s_R	0.77	1.12	0.88	1.25	1.12	1.41
RSD_r, %	2.56	7.37	4.20	2.86	11.90	4.69
RSD_R, %	2.56	10.58	4.20	3.97	11.90	7.19
			Parsley, g DF/100 g			
1	28.59	7.31	28.23	35.54	0.36	21.28
	26.92	6.59	32.90	39.49	5.98	20.33
2	27.09	5.36	29.06	34.42	1.97	21.73
	28.10	5.20	27.46	32.66	0.64	22.90
3	27.81	7.37	25.77	33.14	2.04	20.44
	28.49	7.59	27.06	34.65	1.43	20.90
4	28.51	6.83	26.18	33.01	2.33	21.68
	27.90	6.57	26.24	32.81	1.66	21.33
5	28.43	7.85	24.96	32.81	3.47	20.58
	27.93	6.69	22.72	29.41	5.21	21.24
6	30.81	7.44	25.11	32.55	5.70	23.37
	29.49	7.75	23.33	31.08	6.16	21.74
7	31.61	7.24	26.41	33.65	5.20	24.37
	31.63	7.69	25.68	33.37	5.95	23.94
8	28.51	7.38	21.13	28.51	7.38	21.13
	27.04	7.62	21.40	29.02	5.64	19.42
9	NA[d]	5.67	24.06	29.73	—	—
	—	5.65	24.19	29.84	—	—
10	31.21	8.70	26.03	34.73	5.18	22.51
	30.14	8.97	24.51	33.48	5.63	21.17
11	29.73	6.40	26.78	33.18	2.95	23.33
	29.09	5.62	26.50	32.12	2.59	23.47
$\bar{X}$	28.95	6.98	25.71	32.69	3.08	21.84
s_r	0.72	0.37	1.30	1.31	1.66	0.73
s_R	1.15	1.05	2.62	2.53	3.29	1.36
RSD_r, %	2.49	5.30	5.06	4.01	53.90	3.34
RSD_R, %	3.97	15.04	10.19	7.74	106.82	6.23

[a] Dry weight basis (except prunes, 4.4% water); as-analyzed.
[b] Grubbs tests outlier.
[c] Cochran test outlier.
[d] NA = not analyzed.

ucts was 13.16%. The s_R of SDF values (g/100 g) ranged from 0.02 for apricots to 1.14 for oat bran on an as-is basis and 0.58 for high-fiber cereal to 2.39 for raisins on a dry weight basis. For IDF determinations, the RSD_R was in the range of 1.16% for soy bran to 12.02% for oat bran, with an average of 5.24%. The s_R of IDF values (g/100 g) ranged from 0.02 for apricots to 1.17 for oat bran on an as-is basis, and from 0.28 for prunes to 2.62 for parsley on a dried basis.

Precision of SDF, IDF, and TDF determinations by direct measurements is considered excellent. The s_R of SDF determi-

Table 2. Measures of precision for determining total dietary fiber by independent analysis[a]

Food	Mean, g/100 g	s_r	s_R	RSD_r, %	RSD_R, %	Max. tol. diff., g/100 g	
						r	R
Cereal products							
Barley	12.25	0.36	0.85	2.88	6.89	0.99	2.38
High-fiber cereal	33.73	0.70	0.94	2.08	2.79	1.96	2.63
Oat bran (pretrial)	16.92	1.06	2.06	6.26	12.17	2.97	5.77
Soy bran	67.14	1.01	1.06	1.50	1.58	2.83	2.97
Fruit and vegetables							
Apricots	1.12	0.01	0.01	0.89	0.89	0.03	0.03
Prunes (pretrial)	9.29	0.13	0.40	1.40	4.31	0.36	1.12
Raisins	3.13	0.09	0.15	2.88	4.79	0.25	0.42
Carrots	3.93	0.13	0.13	3.31	3.31	0.36	0.36
Green beans	2.89	0.07	0.07	2.42	2.42	0.20	0.20
Parsley	2.66	0.07	0.14	2.63	5.26	0.20	0.39

[a] As-is (fresh weight basis).

nation (0.02–1.14 g/100 g) is equal to or lower than those of IDF (0.02–1.17 g/100 g) and TDF (0.01–2.06 g/100 g) determinations, even though the RSD_R was higher for SDF values (13.2 vs 4.4–5.2%). Because most foods contain lower amounts of SDF than of IDF, obtaining natural foods containing more than 8 g SDF/100 g for use in this study was difficult.

Tables 1–9 and Figures 1–6 compare the dietary fiber values obtained by direct measurement and by calculation, with respect to mean dietary fiber values and their variation. The measured TDF values by independent analysis and the calculated TDF (C-TDF) values by summing SDF and IDF (C-SDF and C-IDF) were in excellent agreement (Tables 1, 2, 5, and 6; Figure 1). The equation expressing the agreement between the 2 values was C-TDF = 1.003 × TDF, measured, and the correlation coefficient, r^2 of the 2 methods was 0.997 (Figure 1). The assay variabilities of the 2 methods, which were estimated by s_r and s_R, RSD_r, and RSD_R, were comparable (Tables 1, 2, and 5). Both methods showed the mean RSD_R of 4.4–4.5% and demonstrated similar cumulative RSD_R distribution curves (Figure 4). The SDF values also showed good correlation between the calculation and direct measurement methods (Tables 1, 3, and 7; Figure 2). The correlation equation was C-SDF = 0.960 × measured SDF, and r^2 was 0.864 (Figure 2).

The IDF values generated by direct measurement and by calculation also show excellent agreement (Tables 1, 4, and 8; Figure 3). The equation was C-IDF = 0.995 × measured IDF, and r^2 was 0.997 (Figure 3). Calculated SDF and IDF values tend to show higher variability than do measured SDF and IDF values (Figures 5 and 6). The overall respective RSD_r and RSD_R of SDF determinations increased from 7.0 and 13.1% by

Table 3. Measures of precision for soluble dietary fiber determination[a]

Food	Mean, g/100 g	s_r	s_R	RSD_r, %	RSD_R, %	Max. tol. diff., g/100 g	
						r	R
Cereal products							
Barley	5.02	0.40	0.62	8.01	12.29	1.12	1.73
High-fiber cereal	2.78	0.44	0.56	15.83	20.14	1.23	1.57
Oat bran (pretrial)	7.17	0.72	1.14	10.04	15.90	2.02	3.19
Soy bran	6.90	0.30	0.60	4.35	8.70	0.84	1.68
Fruits and vegetables							
Apricots	0.53	0.02	0.02	3.77	3.77	0.06	0.06
Prunes (pretrial)	5.07	0.11	0.31	2.17	6.11	0.31	0.87
Raisins	0.73	0.05	0.16	6.85	21.92	0.14	0.45
Carrots	1.10	0.07	0.18	6.36	16.36	0.20	0.50
Green beans	1.02	0.08	0.11	7.84	10.78	0.22	0.31
Parsley	0.64	0.03	0.10	4.69	15.63	0.08	0.28

[a] As-is (fresh weight) basis.

Table 4. Measures of precision for insoluble dietary fiber determination[a]

Food	Mean, g/100 g	s_r	s_R	RSD_r, %	RSD_R, %	Max. tol. diff., g/100 g	
						r	R
Cereal products							
Barley	7.05	0.61	0.61	8.62	8.62	1.70	1.70
High-fiber cereal	30.52	0.44	0.71	1.44	2.33	1.23	1.99
Oat bran (pretrial)	9.73	0.85	1.17	8.74	12.02	2.38	3.28
Soy bran	60.53	0.70	0.70	1.16	1.16	1.96	1.96
Fruits and vegetables							
Apricots	0.59	0.02	0.02	3.39	3.39	0.06	0.06
Prunes (pretrial)	4.17	0.07	0.09	1.68	2.16	0.20	0.25
Raisins	2.37	0.04	0.07	1.69	2.95	0.11	0.20
Carrots	2.81	0.09	0.16	3.20	5.69	0.25	0.45
Green beans	2.01	0.08	0.08	3.98	3.98	0.22	0.22
Parsley	2.37	0.12	0.24	5.06	10.13	0.34	0.67

[a] As-is (fresh weight) basis.

direct measurement to 18.0 and 27.6% by the difference method (Tables 3, 7, and 9). The overall respective RSD_r and RSD_R of IDF determination are 3.9 and 5.2% for the measured values and 4.6 and 8.7% for the calculated values (Tables 4, 8, and 9).

The variations of calculated SDF values in relation to those of measured SDF values were not consistent. For grains and cereals, the variations of C-SDF values are almost doubled relative to the measured SDF values, although fruits and vegetables (except parsley) showed comparable precision parameters between the 2 methods. For parsley, the variability of C-SDF values is approximately 6–7 times as great as that of measured SDF values, probably because parsley contains a low amount of SDF, and 2 of the IDF values reported are slightly higher than the TDF values. Negative C-SDF values resulted. In reality, the laboratories would report 0 g SDF when the IDF values were higher than the TDF values. However, for estimation of actual variation of C-SDF values, the negative values, instead of 0, were used in the precision estimate. Parsley may contain a low concentration of water-insoluble dietary fiber components that can be solubilized in 78% ethanol. One collaborator suggested that washing IDF residues with portions of ethanol and acetone immediately after separation from the SDF solution could minimize the higher IDF values occasionally encountered for the special commodities such as parsley.

This study shows that the s_R for dietary fiber determination on a dry weight basis is relatively constant. Approximately 80% of the s_R values are in the range of 0.5–1.5 g/100 g across the products. Each food group tended toward its own relatively constant s_R when calculated on an as-is basis. The s_R values for SDF, IDF, and TDF determinations are 0.02–0.31, 0.02–0.24, and 0.01–0.40, respectively, for fruits and vegetables, and 0.56–0.62, 0.62–0.71, and 0.85–1.06, respectively, for cereal and grain products except oat bran. Thus, changing the basis

Table 5. Measures of precision for determining total dietary fiber as a sum of SDF and IDF[a]

Food	Mean, g/100 g	s_r	s_R	RSD_r, %	RSD_R, %	Max. tol. diff., g/100 g	
						r	R
Cereal products							
Barley	12.14	0.39	0.70	3.21	5.77	1.10	1.96
High-fiber cereal	33.30	0.63	0.90	1.89	2.70	1.76	2.52
Oat bran (pretrial)	16.90	0.99	1.49	5.86	8.82	2.77	4.17
Soy bran	67.56	0.56	0.94	0.83	1.39	1.57	2.63
Fruit and vegetables							
Apricots	1.12	0.02	0.02	1.79	1.79	0.06	0.06
Prunes (pretrial)	9.37	0.12	0.30	1.28	3.20	0.34	0.84
Raisins	3.10	0.05	0.18	1.61	5.81	0.14	0.50
Carrots	3.92	0.11	0.13	2.81	3.32	0.31	0.36
Green beans	3.03	0.09	0.12	2.97	3.96	0.25	0.34
Parsley	3.01	0.12	0.23	3.99	7.64	0.34	0.64

[a] As-is (fresh weight) basis.

Table 6. Comparison of measured and calculated TDF (g DF/100 g)[a]

Food	Measured TDF[b]	Calculated TDF (SDF + IDF)[c]
Cereal products		
Barley	12.25	12.14
High-fiber cereal	33.73	33.30
Oat bran	16.92	16.90
Soy bran	67.14	67.56
Fruits and vegetables		
Apricots	1.12	1.12
Prunes	9.29	9.37
Raisins	3.13	3.10
Carrots	3.93	3.92
Green beans	2.89	3.03
Parsley	2.66	3.01

[a] As-is (fresh weight) basis.
[b] Measured total DF is by independent analysis.
[c] Calculated total DF is the sum of soluble and insoluble DF.

from dry weight to as-is makes little difference with dried products such as grain and cereals and improves the precision parameters s_r and s_R for high moisture products such as fruits and vegetables. Relative standard deviations, RSD_r and RSD_R, were not changed by the basis change.

Oat bran showed a higher s_R than did the other grains. Collaborator 5 reported averages of 5.72, 10.95, and 13.90 g/100 g for SDF, IDF, and TDF, respectively, the first time he used this modification. He reported similar averages (5.53, 9.77, and 14.08 g/100 g, respectively) a week later when he used the method of Li and Andrews (23). One month later, however, the laboratory repeated the analysis by this modified method and obtained 16.34 g/100 g for TDF. The values reported the first time were used in the statistical evaluation of the data. The high time-to-time variability difference in values by these methods indicates that sample heterogeneity might contribute to the high assay variability of oat bran. Bran products, especially oat bran, have a natural tendency to separate (15), and the particle size of oat brans is highly variable (24). The data also indicate that oat bran analysis requires an improved method for sample preparation to minimize the heterogeneity problem. Similar observations were made in the AACC Oat Bran Committee Collaborative Study (14).

Overall, the findings in this study show that this dietary fiber determination method is highly compatible with soluble, insoluble, and total dietary fiber labeling on cereal, fruit, and vegetable products. In the case of fruits and vegetables, the method showed excellent performance on products containing dietary fiber as low as 0.53 g/100 g (Table 3). For example, the R value ("maximum tolerable differences") of SDF values for apricot, calculated by $2.8 \times s_R$, was 0.06 when the mean SDF value was 0.53 g/100 g. This finding indicates that 2 values from different laboratories are expected to disagree with each other by no more than R g/100 g, 0.06 g/100 g for apricot, and that 95% of the SDF values may fall into a range of 0.50–0.56 g/100 g. In the case of IDF values for apricot, the R value (g/100 g) was 0.06 when the mean IDF value was 0.59 (Table 4), indicating that 95% of the IDF values might be in the range of 0.56–0.62. The TDF values for apricot (g/100 g) showed even better precision; the R value of 0.06 and the mean TDF value of 1.12 (Table 5) indicated that 19 times in 20 the TDF values might fall into the 1.09–1.15 g range.

The method can also reproducibly measure dietary fiber content as low as 2.78 g/100 g in cereal products. For example, the s_r and s_R of SDF (g/100 g) for high-fiber cereal were 0.44 and 0.56, respectively, when the mean SDF value was 2.78. The R value was 1.57 g/100 g, indicating that 95% of the time the 2 values from different laboratories would be within the range of 2.00–3.57 g/100 g. The s_L (SD among-laboratories) was also 0.33 g/100 g, indicating that in 95% of the cases the mean of 2 DF values from different laboratories would be in the range of 2.32–3.24 g/100 g. This reproducibility value is

Table 7. Measures of precision for C-SDF determination by difference between TDF and IDF[a]

Food	Mean, g/100 g	s_r	s_R	RSD_r, %	RSD_R, %
Cereal products					
Barley	5.13	0.61[b]	0.92[c]	11.84	17.85
High-fiber cereal	3.12	0.98[c]	1.32[c]	31.41	42.31
Oat bran (pretrial)	7.19	1.43[c]	2.11[c]	19.89	29.35
Soy bran	6.38	1.15[c]	1.23[c]	18.03	19.28
Fruits and vegetables					
Apricots	0.53	0.02	0.02	3.77	3.77
Prunes	4.97	0.23[c]	0.38[c]	4.63	7.65
Raisins	0.74	0.08[b]	0.14	10.81	18.92
Carrots	1.12	0.16[c]	0.20	14.29	17.86
Green beans	0.90	0.11	0.11	12.22	12.22
Parsley	0.28	0.15[c]	0.30[c]	53.57	107.14

[a] As-is (fresh weight) basis.
[b] The C-SDF values showed significantly higher variance than did the measured values ($P < 0.10$).
[c] The variance of the C-SDF values was significantly higher than that of the measured SDF values at $P < 0.05$.

Table 8. Measures of precision for C-IDF determination by difference between TDF and SDF[a]

Food	Mean, g/100 g	s_r	s_R	RSD_r, %	RSD_R, %
Cereal products					
Barley	7.20	0.51	0.96[b]	7.08	13.33
High-fiber cereal	30.90	0.68[c]	0.85	2.20	2.75
Oat bran (pretrial)	9.75	1.02	2.32[b]	10.46	23.79
Soy bran	60.14	1.08[c]	1.16[b]	1.80	1.93
Fruits and vegetables					
Apricots	0.61	0.02[c]	0.04[b]	3.28	6.56
Prunes	4.20	0.20[b]	0.50	4.76	11.90
Raisins	2.42	0.09[b]	0.18[b]	3.72	7.44
Carrots	2.83	0.13	0.19	4.59	6.71
Green beans	1.88	0.09	0.13[b]	4.79	6.91
Parsley	2.01	0.07[b]	0.12[b]	3.48	5.97

[a] As-is (fresh weight) basis.
[b] The variance of the C-IDF values was significantly higher than that of the measured IDF values at $P < 0.05$.
[c] The variance of the C-IDF values was significantly higher than that of the measured IDF values at $P < 0.10$.

considered suitable for nutrition labeling of dietary fiber at this low concentration of DF. Official routine practice in Japan (25) has demonstrated that the assay working range can be reduced to as low as 1 g DF/100 g in dried foods, including cereals and grains, by increasing the analytical portion from 1 to 3 g.

Two materials already collaboratively studied by the unmodified AOAC method (11) were used in the present study to compare method performance with regard to mean DF values. Specifically, the same batches of carrot and soy bran from the previous AOAC study (11) were introduced in this study to investigate if this modification could generate mean soluble, insoluble, and total dietary fiber values similar to those obtained by the unmodified methods. As shown in Table 10, the 2 methods generate remarkably similar mean SDF, IDF, and C-TDF values, even though the 2 studies used different participating laboratories at different times.

Agreement of the dietary fiber values for soy bran by the 2 methods was within 0.53 g/100 g. The unmodified method and the modification generated values (g/100 g) of 6.62 and 6.90 for SDF, 61.00 and 60.53 for IDF, and 67.62 and 67.56 for TDF, respectively. Agreement on values for carrots was also excellent, within 0.12 g DF/100 g. The values (g/100 g) obtained by the method and the modification were 0.98 and 1.10 for SDF, 2.28 and 2.81 for IDF, and 3.86 and 3.92 for TDF, respectively. The modified method showed better precision in most cases than the earlier method. With the modification, the s_R for soy bran was reduced by 41% and 68% for SDF and TDF, respectively; the s_R for carrot was reduced by 50% for IDF. The RSD_R of SDF values for carrot were comparable between the 2 methods. The comparison of precision of the unmodified method and the present modification was further extended to other products listed in Table 11. We emphasize that the 2 studies used different participating laboratories at different times. The modification showed significant precision improvements for determination of SDF, IDF, and TDF (by summing SDF and IDF) on most products tested here, especially for fruits and parsley.

From the comparative data in Tables 10–11, we surmised that neither the simplification of the method nor the buffer change altered the mean DF values. These modifications could further improve the precision of method performance, even for the products that showed good precision with the unmodified

Table 9. Summary of statistical parameters for dietary fiber determination[a]

DF type	Determination method	Concn range, g/100 g	RSD_R av., %	RSD_R range, %
SDF	Direct measurement	0.53–7.2	13.16	3.8–21.9
	Calculation (TDF – IDF)	0.28–7.2	18.81[b]	7.6–42.4
			(27.67)	(7.6–107.1)
IDF	Direct measurement	0.59–60.5	5.24	1.2–12.0
	Calculation (TDF – SDF)	0.61–60.1	8.73	1.9–23.8
TDF	Direct measurement	1.1–67.1	4.44	0.9–12.2
	Calculation (SDF + IDF)	1.1–67.6	4.45	1.4–8.8

[a] As-is (fresh weight) basis.
[b] The average RSD_R without parsley data. The number in parentheses includes parsley data.

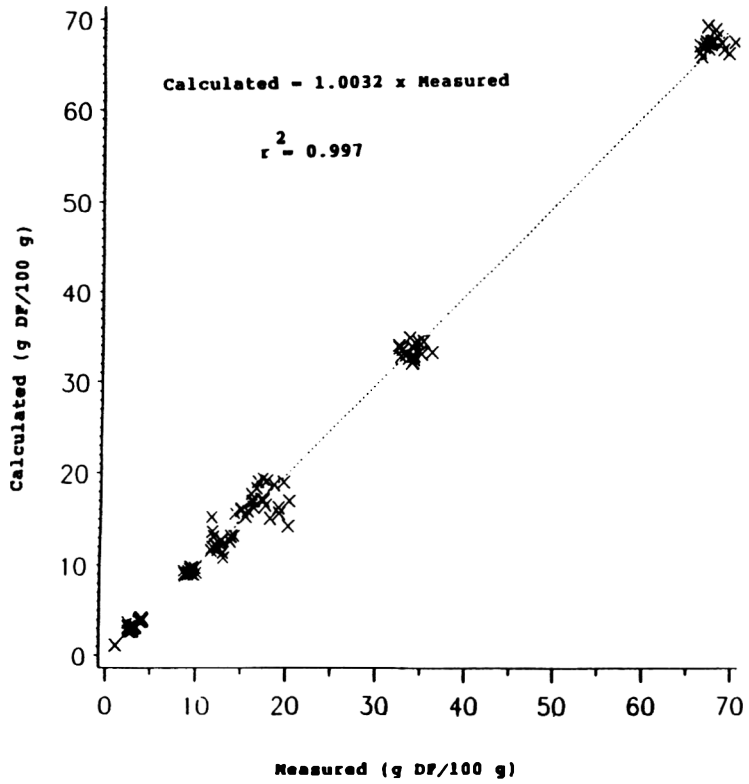

Figure 1. Correlation of measured and calculated TDF values.

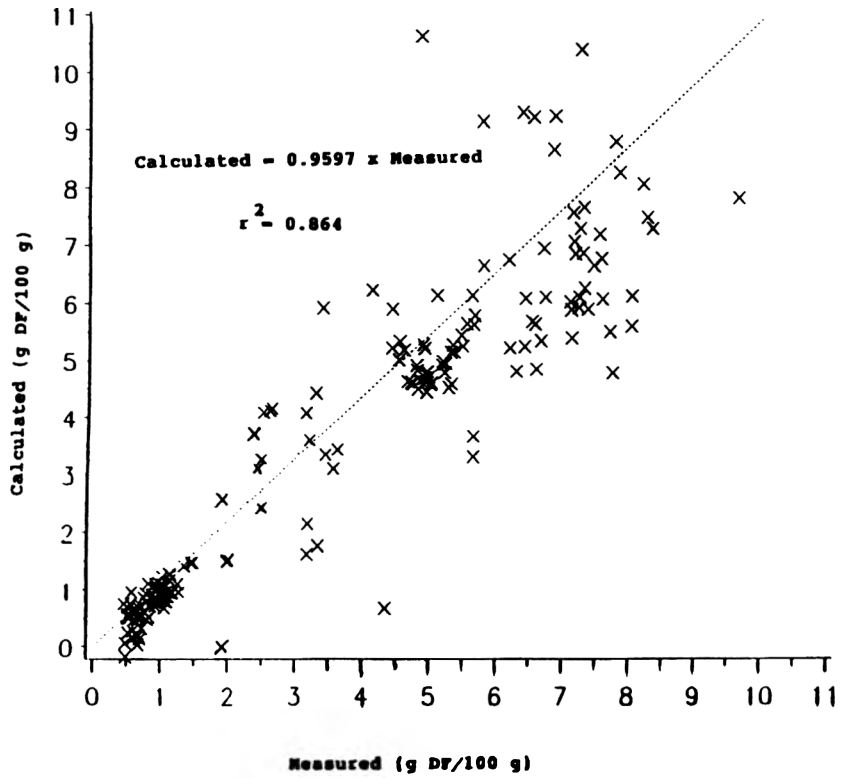

Figure 2. Correlation of measured and calculated SDF values.

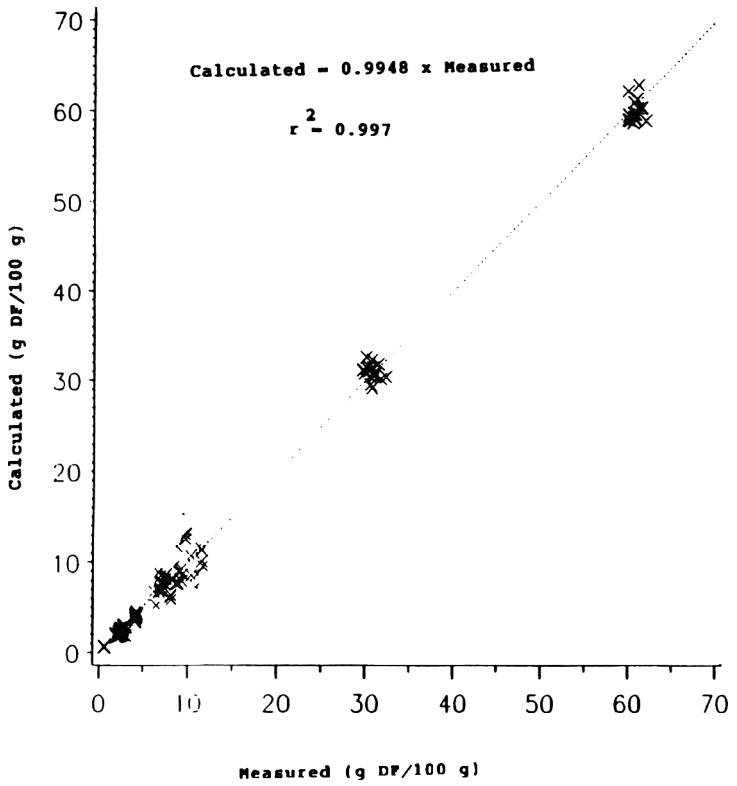

Figure 3. Correlation of measured and calculated IDF values.

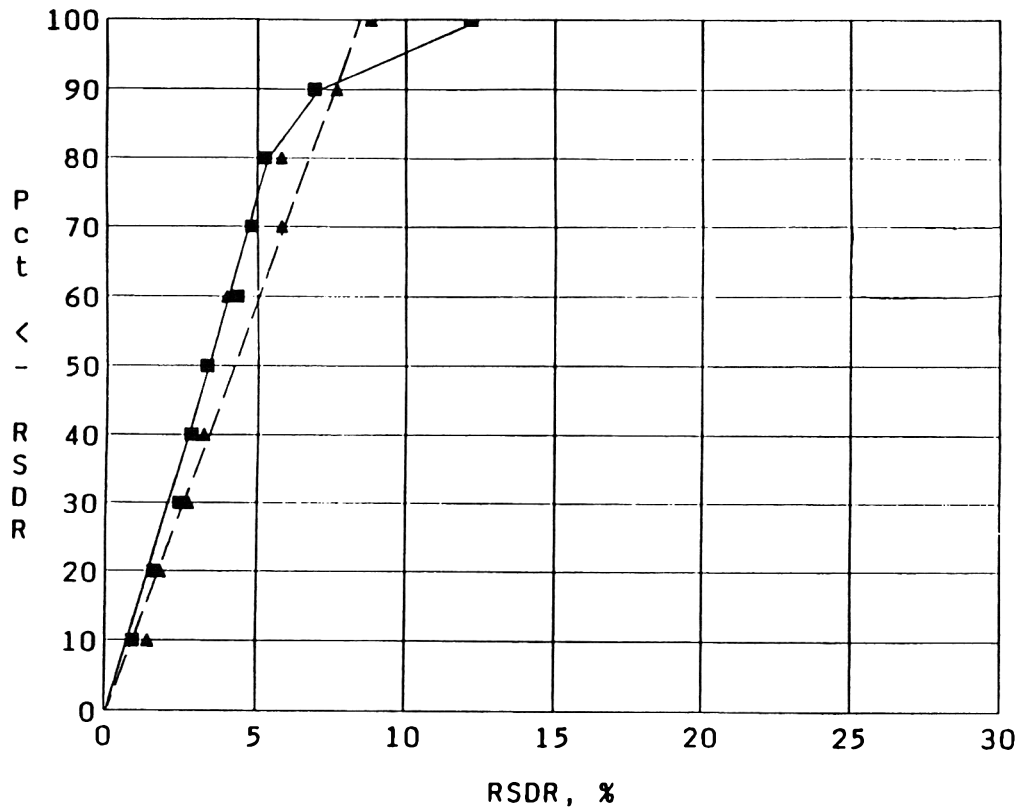

Figure 4. Comparison of cumulative RDS_R for TDF determinations: a, by direct measurements ■; b, by difference (TDF–IDF) ▲.

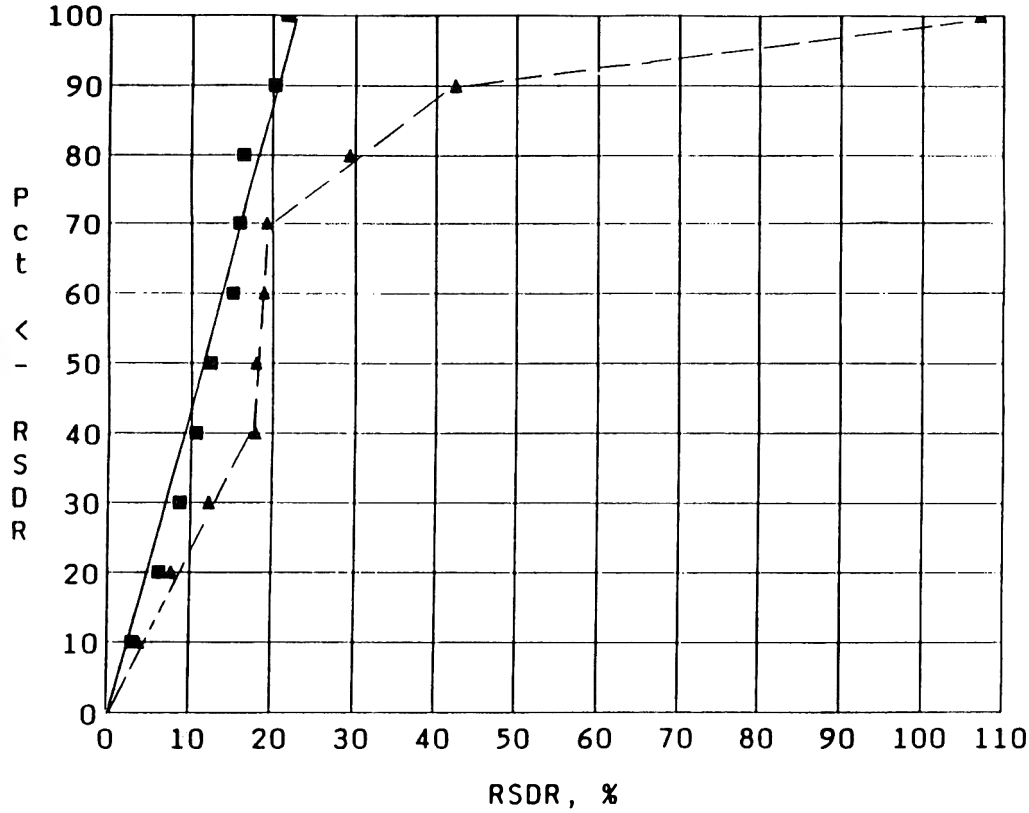

Figure 5. Comparison of cumulative RSD_R for SDF determinations: a, by direct measurements ■; b, by difference (TDF-SDF) ▲.

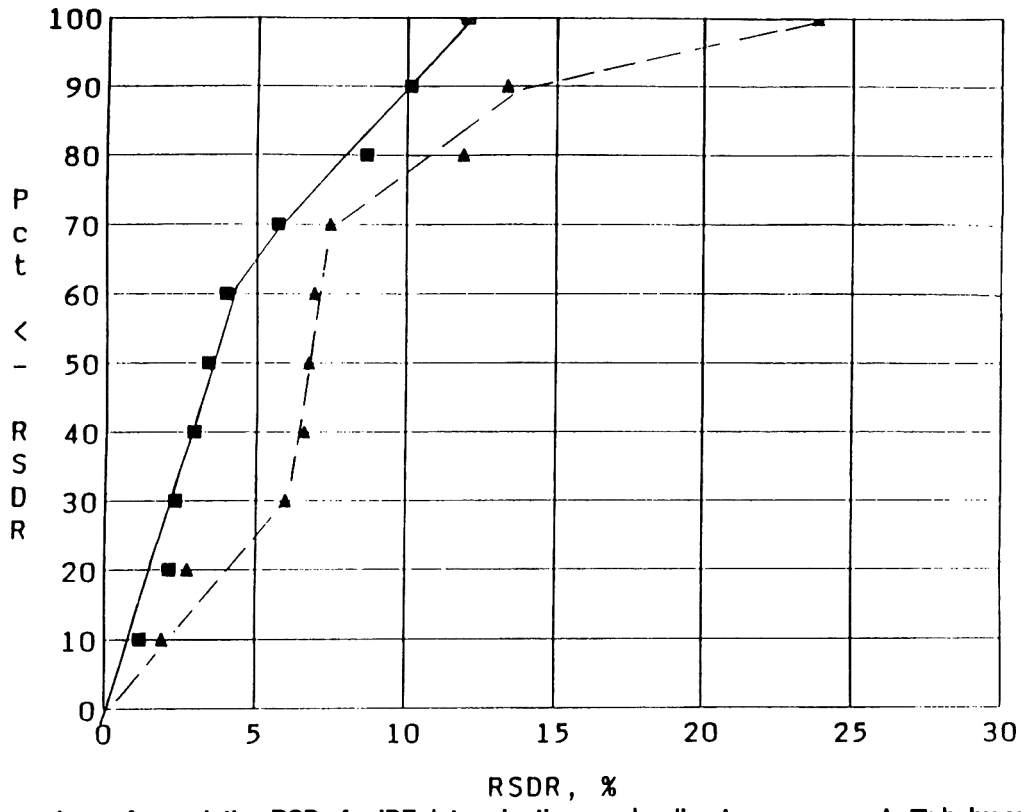

Figure 6. Comparison of cumulative RSD_R for IDF determinations: a, by direct measurements ■; b, by summation (SDF + IDF) ▲.

Table 10. Method performance of 2 dietary fiber determination methods[a]

Statistic	SDF		IDF		TDF (SDF + IDF)	
	Unmod.[b]	Mod.[c]	Unmod.	Mod.	Unmod.	Mod.
	Dry weight basis (as-analyzed)					
Soy bran						
$\bar{X}$, g/100 g	7.08	7.38	65.24	64.74	72.68	72.25
s_R, g/100 g	1.04	0.64	2.40	0.75	1.78	1.00
Carrots						
$\bar{X}$, g/100 g	11.02	12.40	32.29	31.61	44.10	44.01
s_R, g/100 g	1.74	2.04	3.68	1.79	2.62	1.42
	As-is (fresh weight) basis					
Soy bran						
$\bar{X}$, g/100 g	6.62	6.90	61.00	60.53	67.62	67.56
s_R, g/100 g	0.97	0.60	2.24	0.70	1.66	0.94
RSD_R, %	14.66	8.70	3.68	1.16	2.45	1.39
Carrots						
$\bar{X}$, g/100 g	0.98	1.10	2.88	2.81	3.86	3.92
s_R, g/100 g	0.16	0.18	0.33	0.16	0.23	0.13
RSD_R, %	15.76	16.36	11.39	5.69	5.94	3.32

[a] Data for the unmodified (1989) and the modified (1990) methods are based on the results from the same batches of test samples by different participating laboratories.
[b] Unmodified method.
[c] Modified method.

method. The precision of the enzymatic-gravimetric method has been greatly improved through the several modifications, including the simplification of the assay protocol and the use of organic buffers. The method might be further improved through the optimization of analytical portion size for each food category (0.5–3.0 g), test sample preparation (optimized uniform particle sizing), and further analytical simplification of the determination. Significant improvements made to date might also be further advanced by the development of optimized determination methods that are physiologically relevant to soluble/insoluble dietary fiber.

Conclusions

(*1*) The modification does not alter mean dietary fiber values, when compared to those for the unmodified method.

(*2*) The precision of enzymatic-gravimetric methods has been significantly improved, and a reliable method for simultaneous determination of soluble, insoluble, and total dietary fiber is now available.

(*3*) The overall precision of the soluble, insoluble, and total dietary fiber determination by direct measurement is excellent.

(*4*) The total dietary fiber values calculated by summing SDF and IDF were in excellent agreement with the TDF values measured independently, and the assay variations by the 2 methods were comparable.

(*5*) The soluble and insoluble dietary fiber values obtained by direct measurement were more consistent and precise than those estimated by the difference method, although the calculated SDF and IDF values also showed good precision, with the exception of C-SDF values for parsley.

(*6*) From a practical point of view, this method for soluble, insoluble, and total dietary fiber determination can be successfully used to generate reliable values for quality control, research, and labeling.

Collaborators' Comments

General comments by the collaborators confirmed that the method was simpler and less time-consuming than the unmodified AOAC method. Many laboratories encountered filtration difficulties with desugared fruits (prunes, raisins, and apricots). Some difficulty was encountered with carrots. When the analytical portion size was reduced from 1.0 to 0.5 g, suspensions of these foods could be filtered faster. Collaborators in research laboratories preferred to use Celite Analytical Filter Aid (CAFA), instead of Celite 545 AW, because CAFA allowed a minimum loss during filtration. However, most of the laboratories in which a multitude of test samples are analyzed daily supported the use of Celite 545 AW because of its fast filtration rate compared to that for CAFA.

Table 11. s_R (g/100 g) of 2 dietary fiber determination methods[a]

Food	SDF		IDF		C-TDF (SDF + IDF)	
	Unmod.[b]	Mod.[c]	Unmod.	Mod.	Unmod.	Mod.
			Dry weight basis (as-analyzed)			
Barley	1.37	0.66	0.62	0.65	1.87	0.75
High-fiber cereal	0.65	0.58	1.31	0.73	1.53	0.93
Oat bran	1.25	1.23	2.06	1.26	2.46	1.61
Apricots	4.31	0.93	3.69	1.02	4.82	1.17
Prunes	9.53	1.02	8.98	0.28	2.99	0.98
Raisins	6.02	2.39	9.49	1.08	7.22	2.70
Parsley	2.92	1.05	4.69	2.62	2.61	2.53
			As-is (fresh weight) basis			
Barley	1.21	0.62	0.55	0.62	1.65	0.70
High-fiber cereal	0.65	0.56	1.27	0.71	1.49	0.90
Oat bran	1.15	1.14	1.90	1.17	2.26	1.49
Apricots	0.25	0.02	0.19	0.02	0.28	0.02
Prunes	2.08	0.31	1.96	0.09	0.65	0.30
Raisins	0.40	0.16	0.63	0.07	0.48	0.18
Parsley	0.34	0.10	0.55	0.24	0.31	0.23

[a] Data for the 2 methods were based on results from different batches of test samples by different participating laboratories.
[b] Data for the unmodified method were calculated from the previous study results (8, 11, 14). High fiber cereal data were from the 1988 AOAC study (11), oat bran data were from the 1989 AACC study (14), and all other data were from the 1989 AOAC study (8).
[c] Modified method.

Recommendations

We recommend that the methods for the determination of soluble, insoluble, and total dietary fiber (by independent analysis and by summing SDF and IDF) be adopted first action. Specifically, we recommend that total dietary fiber values be determined either by summing soluble and insoluble dietary fiber values, or by independent analysis.

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DRUG FORMULATIONS

^{1}H NMR Spectroscopic Method with Chiral Eu(III) Shift Reagent for the Determination of the Enantiomeric Composition of Naproxen

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A simple method based on the use of ^{1}H NMR spectroscopy with chiral Eu(III) shift reagent is described for the determination of (*S*)-(+)- and (*R*)-(−)-naproxen in the presence of each other. To enhance the coordinating affinity of the substrate for the lanthanide ion, the sample was first derivatized to a mixture of methyl esters, which in the presence of Eu(hfc)$_3$ formed short-lived diastereomeric complexes with sufficient nonequivalency in the ^{1}H NMR spectrum. Optimum complexing conditions corresponded to concentrations of substrate and Eu(hfc)$_3$ of 0.1M each in $CDCl_3$. In this matter, the enantiomeric ester-methyl protons and α-methyl protons yielded well-resolved resonance signals of utility in the measurement of enantiomeric compositions. Recovery studies demonstrated that the proposed method is quantitative.

Naproxen, 6-methoxy-α-methyl-2-naphthaleneacetic acid, is a systemic nonsteroidal anti-inflammatory and analgesic agent widely used for the relief of the symptoms of acute and chronic rheumatoid arthritis, osteoarthritis, juvenile arthritis, and acute gout (1–3). The presence of a chiral carbon adjacent to its carboxyl functional group permits this compound to exist as both *rectus* (*R*) and *sinister* (*S*) enantiomers (4). Like other arylacetic acid derivatives, the pharmacological potencies of the optical antipodes of naproxen are known to be different and to reside mostly in the (*S*)-(+)-enantiomer (1, 3–7). Thus, (*S*)-(+)-naproxen has shown a much greater activity than the (*R*)-(−)-antipode on both platelet aggregation and prostaglandin (thromboxane B_2) synthesis from collagen-stimulated human platelets (4) and on laboratory models of inflammation (1, 3).

The stereoselective chromatographic separation of the enantiomers of naproxen and of related arylacetic acids with analgesic and anti-inflammatory actions has been the subject of several reports (8–14). For example, the enantiomers of ibuprofen (8) and indoprofen (9) have been determined as the α-methylbenzylamide derivatives by gas chromatography (GC), and the same type of derivative has been used for the analysis of benoxaprofen (10) and carprofen (7) by liquid chromatography (LC). In addition, the enantiomers of naproxen have been analyzed as a pair of diastereomers by LC on achiral columns after acid-catalyzed esterification with (*S*)-(+)-2-octanol (11) or after amide formation with either (−)-1-(4-dimethylamino-1-naphthyl)ethylamine (12) or (*S*)-(−)-1-phenylethylamine (13). An alternative approach entails conversion of the enantiomers of naproxen to a pair of diastereomeric 1-naphthalenemethylamides before LC separation on a chiral stationary phase (14). Although chiral derivatization of a mixture of diastereomers can lead to a most satisfactory enantioselective resolution, this approach is susceptible to drawbacks such as the possibility of partial racemization during the derivatization reaction (8, 11) or kinetic resolution due to (*1*) differences in diastereomeric transition states (11, 14); (*2*) the likelihood of introducing systematic error upon the use of an enantiomerically impure chiral derivatizing reagent (11, 15); (*3*) long analysis times (15); (*4*) the necessity for multiple procedural steps that may result in significant sample losses; and (*5*) the reliance on samples of the pure enantiomers for use as reference standards during the quantification steps.

The purpose of this paper is to report the development of a simple, straightforward, and accurate ^{1}H NMR spectroscopic method to determine the optical purity of naproxen. The proposed method is based on the formation of a pair of short-lived and reversible diastereomeric solvates displaying resonances that are sufficiently separated in the ^{1}H NMR spectrum of the sample mixture so that the enantiomeric levels can be measured without reference to an enantiomerically pure external standard.

Experimental

Apparatus and Reagents

(a) *Spectrometer.*—All ^{1}H NMR spectra were recorded on a 90 MHz Varian EM-390 spectrometer (Varian Instruments, Palo Alto, CA) operating at a probe temperature of 35 ± 1°C.

(b) *Reference standard.*—Tetramethylsilane (TMS, Aldrich Chemical Co., Milwaukee, WI), washed first with con-

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Table 1. Determination of the enantiomeric composition of synthetic mixtures of (*S*)-(+)- and (*R*)-(−)-naproxen by ^{1}H NMR spectroscopy with Eu(hfc)$_3$[a]

			(*S*)-(+)-enantiomer, %				
			-C-CH$_3$			-CO$_2$CH$_3$	
Sample	(*R*)-(−)-enantiomer, mg	(*S*)-(+)-enantiomer, mg	Added	Found	Rec., %[b]	Found	Rec., %
1	113.31	2.22	1.92	1.88	97.92	1.89	98.44
2	9.11	2.35	20.51	20.35	99.22	20.41	99.51
3	7.93	3.55	30.92	30.81	99.64	30.88	99.87
4	6.75	4.78	41.46	41.75	100.70	41.65	100.46
5	6.10	5.39	46.19	46.63	99.40	46.72	99.59
6	4.55	6.95	60.43	60.33	99.83	60.21	99.64
7	4.08	7.45	64.61	64.85	100.37	64.42	99.71
8	3.01	8.53	73.92	73.39	99.28	73.29	99.15
9	2.27	9.25	80.30	80.01	99.63	80.07	99.71
10	1.62	9.93	85.97	84.15	97.88	84.39	98.16
Mean					99.39		99.42
SD					0.92		0.68

[a] Total concentration of drug was 0.1M in $CDCl_3$, and the Eu(hfc)$_3$–substrate molar ratio was 1.0.
[b] Recoveries were calculated from (amt found × 100)/amt added. Amt found, mg S(+), was calculated from [AS(+)/AS(+) + AR(−)] × mg sample taken.

centrated sulfuric acid and next with saturated potassium bicarbonate, distilled, and stored over type 4A molecular sieves (Aldrich Chemical Co.).

(c) *Deuterated chloroform ($CDCl_3$).*—Isotopic purity, +99.5% (Aldrich Chemical Co.), distilled before use, and stored over type 4A molecular sieves.

(d) *Chiral shift reagent [Eu(hfc)$_3$].*—Tris[3-heptafluoropropylhydroxymethylene)-(+)-camphorato]europium(III) (Aldrich Chemical Co.), stored over P_2O_5 in an evacuated desiccator (or under dry nitrogen). All experiments with Eu(hfc)$_3$ were conducted under conditions that would minimize the possibility of contamination by ambiental moisture or air, i.e., within a glove box and under dry nitrogen.

(e) *Samples.*—(*S*)-(+)- and (*R*)-(−)-naproxen were generously supplied by the manufacturer (Syntex Laboratories, Palo Alto, CA).

Preparation of Samples

Synthetic mixtures of (*S*)-(+)- and (*R*)-(−)-naproxen were prepared by accurately weighing the quantities of each enantiomer that are listed in Table 1. These samples were first converted to the corresponding methyl esters by either of the following methods:

(a) *Refluxing method.*—The sample was dissolved in 40 mL methanol, mixed with 2 mL 12M hydrochloric acid, and refluxed 1 h. The reaction mixture was evaporated to a small volume under reduced pressure, transferred to a separatory funnel, and extracted with three 15 mL portions of ether. The ethereal extracts were combined, the solvent was evaporated to dryness under a stream of dry nitrogen, and the residue was dried at 50°C *in vacuo*.

(b) *Diazomethane treatment.*—The sample was allowed to react with 3 mL freshly prepared 0.25M ethereal diazomethane for 5 min at room temperature. Then, the solution was evaporated to dryness under a stream of dry nitrogen, and the residue was dried at 50°C *in vacuo*. Solutions for ^{1}H NMR studies were prepared by dissolving the residue of methyl esters in $CDCl_3$ containing 1% (v/v) TMS. These solutions were stored immediately in glass vials that were crimper-sealed with Teflon-coated rubber septa and aluminum seals. Samples for analysis were withdrawn through the septa by means of a fixed needle, liquid-tight, dry microliter syringe.

NMR Studies of Lanthanide-Induced Shifts

The required changes in lanthanide shift reagent to substrate (L/S) molar ratios were obtained by first adding the shift reagent to a dry NMR tube and then adding the appropriate aliquot of substrate stock solution (the exact amount having been determined gravimetrically). The NMR tube was capped immediately, and its contents were mixed by inversion, allowed to stand 10 min, and then placed in the spectrometer for recording the ^{1}H NMR spectrum. A second aliquot of the substrate stock solution was added to the same tube, and the spectrum was recorded once more. The additions and spectral recordings were repeated until an appropriate number of spectra were available for properly defining the effects of the molar ratio of L/S on the enantiomeric spectral lines.

Determination of Enantiomeric Purities

An accurately weighed quantity of naproxen sample (ca 11.5 mg) was converted to the methyl ester as described under *Preparation of Samples*. The dry residue was dissolved in 0.5 mL $CDCl_3$ containing 1% (v/v) TMS, and the solution was

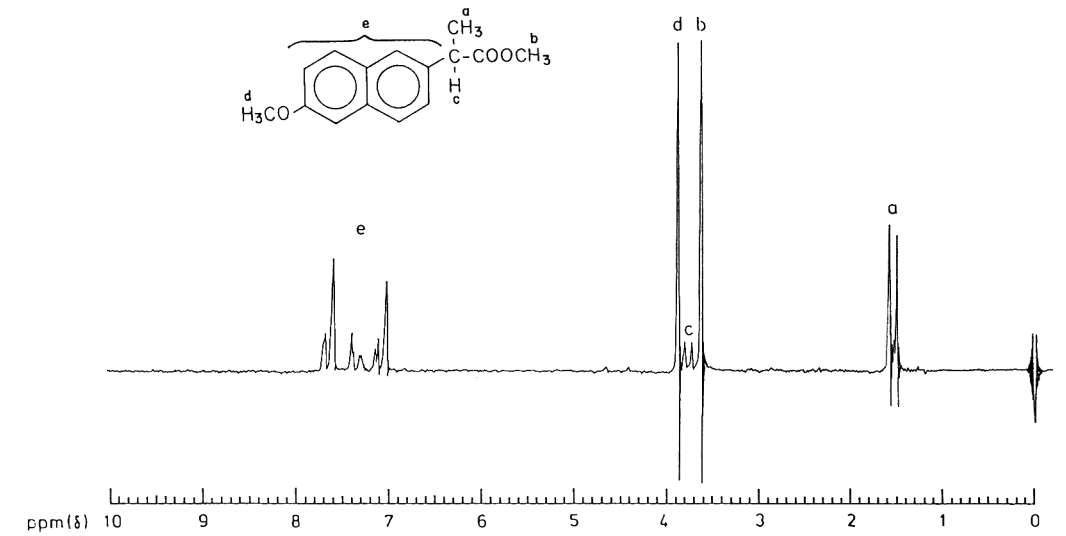

Figure 1. ^{1}H NMR spectrum of a mixture of (*R*)-(−)- and (*S*)-(+)-naproxen methyl esters in $CDCl_3$.

transferred to a dry NMR tube containing ca 59.5 mg $Eu(hfc)_3$. The tube was capped, inverted several times to effect solution, and allowed to stand 10 min; the ^{1}H NMR spectrum of the solution was then recorded. The relative intensities of the resonance signals (peak heights or peak areas) for the enantiomeric ester-methyl protons (singlets) at 9.03 ppm [(*S*)-(+)-enantiomer] and 9.21 ppm [(*R*)-(−)-enantiomer] or, alternatively, the enantiomeric signals for the α-methyl protons (doublets) at 5.87 ppm [(*S*)-(+)-enantiomer] and 5.67 ppm [(*R*)-(−)-enantiomer] were measured and used to calculate the percentage of each enantiomer in the sample taken from the following equations:

$$\%\ (S)\text{-}(+)\text{-enantiomer} = [AS(+) \times 100]/[AS(+) + AR(-)], \text{ and}$$
$$\%\ (R)\text{-}(-)\text{-enantiomer} = [AR(-) \times 100]/[AS(+) + AR(-)]$$

where $AS(+)$ = peak area (or peak height) of the resonance signal for the (*S*)-(+)-enantiomer, and $AR(-)$ = peak area (or peak height) of the resonance signal for the (*R*)-(−)-enantiomer.

Results and Discussion

The extent of the lanthanide-induced shift is strongly influenced by the lanthanide–substrate complexation binding constants. Because the lanthanide shift reagent is a Lewis acid, the complexation binding constant of the adduct is a function of substrate basicity (16–21). Of the 2 functional groups capable of coordinating with the lanthanide ion, namely, the aryl methoxy group and the carboxyl group, the former functionality will complex less effectively with the lanthanide shift reagent than the latter because of its much lower affinity (22). However, because of the known instability of complexes of a lanthanide shift reagent with substrates containing carboxyl groups (19, 20), the enantiomers of naproxen were first converted into the methyl ester derivatives. Like other arylacetic acid derivatives, esterification with either methanolic hydrochloric acid or ethereal diazomethane was rapid and quantitative and afforded a product of such purity that it did not require purification (23, 24). Ester groups demonstrate enhanced coordinating ability by virtue of their appreciable Lewis basicity and their minimal steric hindrance (22, 25, 26). The ^{1}H NMR spectra of (*S*)-(+)- and (*R*)-(−)-naproxen methyl esters in $CDCl_3$ shown in Figure 1 displayed the following resonances: (a) a doublet at 1.53 ppm (α-methyl proton); (b) a singlet at 3.62 ppm (ester methyl protons); (c) a quartet centered at 3.77 ppm (α-methine proton); (d) a singlet at 3.87 ppm (methoxy protons); and (e) multiplets in the region 7.0–7.8 ppm (2,6-disubstituted naphthalene).

The ^{1}H NMR spectra of a mixture of (*S*)-(+)- and (*R*)-(−)-enantiomers of naproxen methyl esters (0.1M in $CDCl_3$) that had been complexed with $Eu(hfc)_3$ at various L/S molar ratios are shown in Figure 2. The large downfield shifts exhibited by the resonances from their original position in the uncomplexed spectrum are induced by the paramagnetic ion mainly as a result of dipole-dipole "through space" interactions between its unpaired electron and the protons being examined (27). Because the equilibrium between the substrate and the lanthanide chelate is rapid on the NMR time scale (28–30), the resulting spectrum will be an average of the spectra of complexed and uncomplexed substrate. The lanthanide-induced shifts (Δδ) for the resonance frequency of a particular proton were found to decrease as the distance from the proton in question to the ester group increased. Accordingly, the largest Δδ was observed with the α-methine proton and the smallest one with the methoxy protons. In most cases, pseudocontact shifts are known to reflect the distance, and the angle between a particular proton and the lanthanide ion as given by the McConnell-Robertson equation (31). Moreover, other contact interactions contributing to some proton resonances may come from protons close to the coordination site (32, 33).

The Δδ values increased with increasing L/S molar ratios. The plots of Δδ for the α-methyl and ester methyl protons vs L/S molar ratios, shown in Figure 3, indicate that the relation-

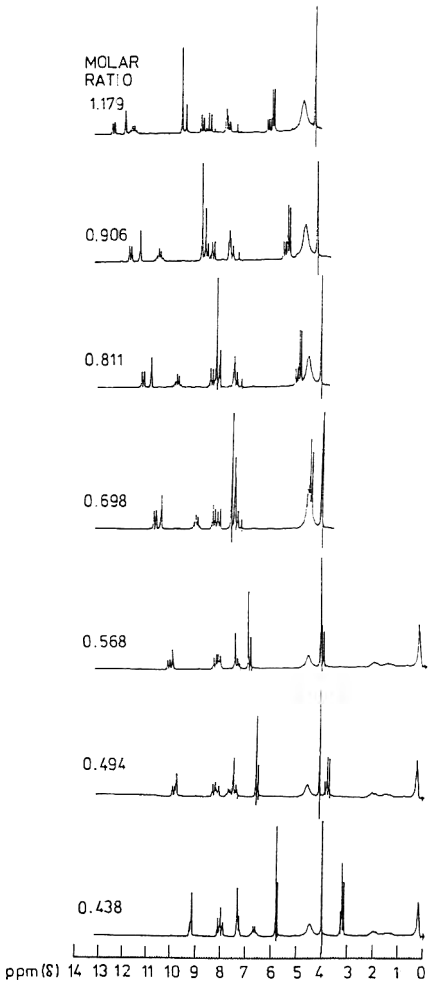

Figure 2. ^{1}H NMR spectra of a mixture of (*R*)-(−)- and (*S*)-(+)-naproxen methyl esters, 0.1M in $CDCl_3$, after complexation with various Eu(hfc)$_3$ to substrate molar ratios.

ship becomes nonlinear at L/S ratios higher than about 0.5. This would suggest that although other than a 1:1 equilibrium may be involved, 1:1 complexes are the predominant ones, because a bend in the curve occurred at an L/S ratio of 1.0. The slopes of the curves differed at low lanthanide reagent concentrations for different substrate concentrations despite equal L/S ratios (21). The optimal substrate concentration at which the chiral lanthanide reagent did not produce any line broadening was about 0.1M.

The differences in induced chemical shifts ($\Delta\Delta\delta$) for the 2 enantiomers after complexation with the chiral lanthanide shift reagent might arise from at least 2, probably mutually dependent, interactions: the differences in equilibrium constants for formation of the various possible diastereomeric complexes between enantiomeric substrates and the chiral lanthanide reagent and the distinct geometries of the resulting complexes. The data presented in Table 2 provide qualitative support for the contributions of these 2 types of interactions to the observed $\Delta\Delta\delta$ values. Additionally, the magnitudes of $\Delta\Delta\delta$ varied with the changes in L/S molar ratios, as seen from the plot of the $\Delta\Delta\delta$ values for the α-methyl and ester-methyl protons vs the L/S ratios shown in Figure 4. Interestingly, the signal for the α-methyl protons of the (*S*)-(+)-enantiomer was shifted to a greater extent than that of the (*R*)-(−)-enantiomer, whereas the reverse situation was noted for the enantiomeric ester methyl signals. Such differences in the sense of nonequivalence are probably a reflection of differences in the geometries of the complexes formed. They clearly demonstrate that the $\Delta\Delta\delta$ values are not simply the result of differences in equilibrium constants but also of differing structural and conformational features for each of the enantiomer-shift chelate complexes. However, in light of the present results, it is not possible to establish their relative contributions to enantiomeric shift differences.

The degree of nonequivalence of the enantiomeric α-methyl doublets and ester-methyl singlets was sufficiently

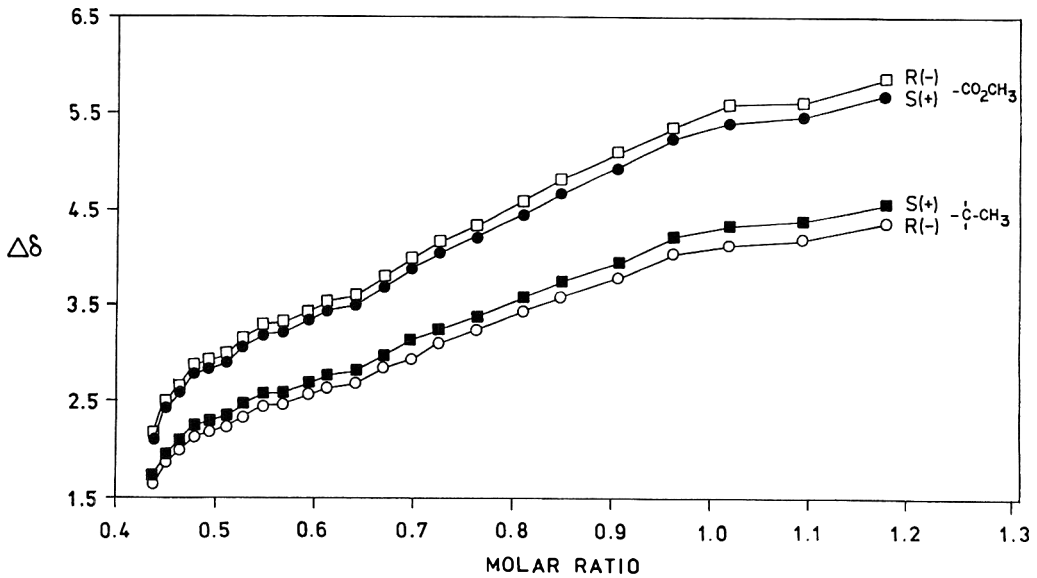

Figure 3. Plot of induced chemical shifts (Δδ) for the -CCH_3 and -CO_2CH_3 protons of (*R*)-(−)- and (*S*)-(+)-naproxen methyl esters vs Eu(hfc)$_3$ to substrate molar ratios.

Table 2. Shift data (ppm) of the -CCH_3 and -CO_2CH_3 protons of (*S*)-(+)- and (*R*)-(-)-naproxen methyl esters after complexation with various molar equivalents of Eu(hfc)$_3$

	-C-CH_3					-CO_2CH_3				
	(*S*)-(+)		(*R*)-(-)			(*S*)-(+)		(*R*)-(-)		
Eu(hfc)$_3$: substrate ratio	δ	Δδ	δ	Δδ	ΔΔδ	δ	Δδ	δ	Δδ	ΔΔδ
1.179	6.10	4.57	5.90	4.38	0.19	9.32	5.70	9.48	5.86	0.16
1.094	5.92	4.39	5.73	4.20	0.19	9.09	5.47	9.25	5.61	0.16
1.019	5.87	4.34	5.67	4.14	0.20	9.03	5.41	9.21	5.59	0.18
0.962	5.75	4.22	5.57	4.04	0.18	8.83	5.24	8.98	5.36	0.15
0.906	5.48	3.95	5.32	3.79	0.16	8.57	4.95	8.72	5.10	0.15
0.849	5.28	3.75	5.13	3.60	0.15	8.31	4.69	8.45	4.83	0.14
0.811	5.12	3.59	4.97	3.44	0.15	8.08	4.46	8.22	4.60	0.14
0.764	4.91	3.88	4.77	3.24	0.14	7.85	4.23	7.97	4.35	0.12
0.726	4.78	3.25	4.64	3.11	0.14	7.68	4.06	7.79	4.17	0.11
0.698	4.67	3.14	4.52	2.99	0.13	7.51	3.89	7.62	4.00	0.11
0.670	4.51	2.98	4.38	2.85	0.13	7.32	3.70	7.43	3.81	0.11
0.642	4.35	2.82	4.22	2.69	0.13	7.12	3.50	7.22	3.60	0.10
0.613	4.30	2.77	4.17	2.64	0.13	7.06	3.44	7.16	3.54	0.10
0.594	4.22	2.69	4.10	2.57	0.12	6.46	3.34	7.05	3.43	0.09
0.568	4.12	2.59	4.00	2.47	0.12	6.85	3.23	6.94	3.32	0.09
0.548	4.10	2.57	3.99	2.46	0.11	6.82	3.20	6.91	3.29	0.09
0.528	4.00	2.47	3.88	2.35	0.10	6.69	3.07	6.71	3.15	0.08
0.511	3.88	2.35	3.78	2.25	0.10	6.53	2.91	6.61	2.99	0.08
0.494	3.83	2.30	3.73	2.20	0.10	6.47	2.85	6.55	2.93	0.08
0.479	3.78	2.25	3.67	2.14	0.09	6.41	2.79	6.49	2.87	0.08
0.464	3.62	2.09	3.53	2.00	0.09	6.20	2.58	6.27	2.65	0.07
0.451	3.47	1.94	3.40	1.87	0.07	6.05	2.43	6.11	2.49	0.06
0.438	3.24	1.71	3.17	1.64	0.07	5.75	2.12	5.80	2.18	0.05

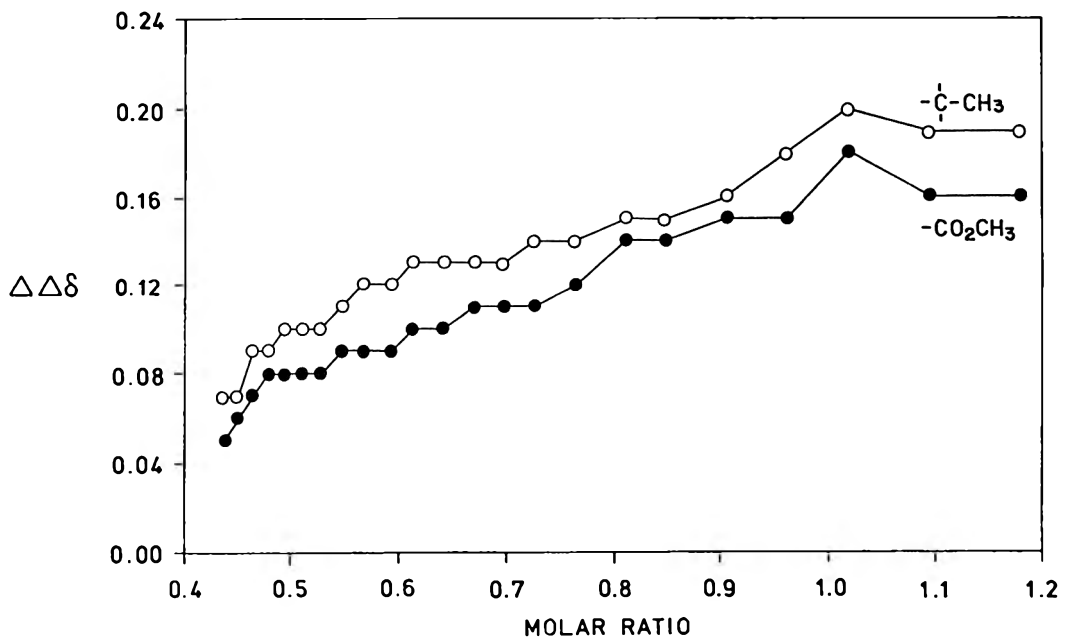

Figure 4. Plot of chemical shift differences (ΔΔδ) for the -CCH_3 and -CO_2CH_3 protons of (*R*)-(-)- and (*S*)-(+)-naproxen methyl esters vs Eu(hfc)$_3$ to substrate molar ratios.

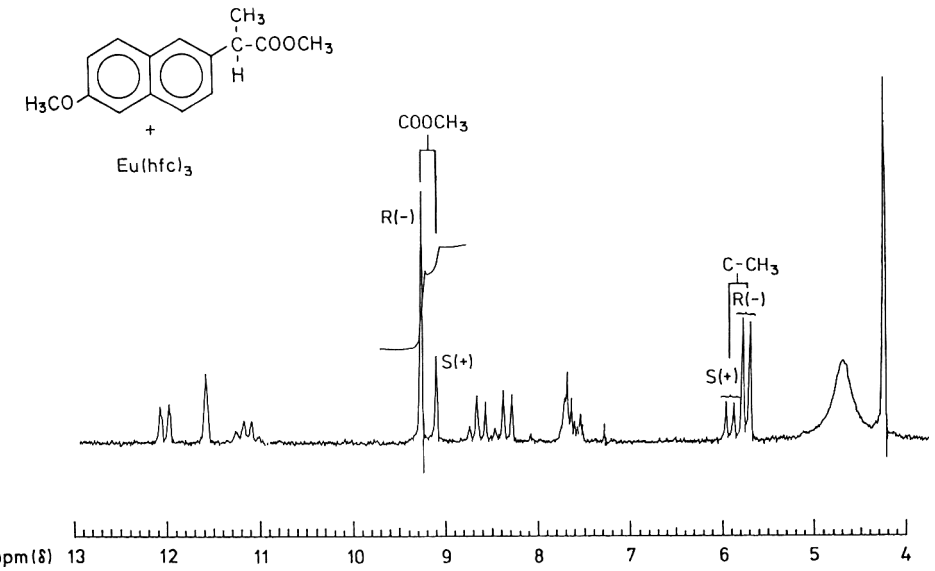

Figure 5. ^{1}H NMR spectrum of a mixture of (*R*)-(−)- and (*S*)-(+)-naproxen methyl esters, 0.1M in $CDCl_3$, after complexation with 1.0M equivalents of Eu(hfc)$_3$.

large and well separated from other signals to permit their use in the direct quantitative determination of the enantiomers. Resolution was optimal at an L/S ratio of about 0.1 in $CDCl_3$. Under these conditions, the enantiomeric α-methyl protons of the (*S*)-(+)- and (*R*)-(−)-enantiomers each resonated as a doublet centered at 5.87 ppm and 5.67 ppm, respectively, whereas the enantiomeric ester-methyl signals of the (*S*)-(+)- and (*R*)-(−)-enantiomers each appeared as a singlet at 9.03 ppm and 9.21 ppm, respectively (Figure 5). Both sets of signals were found to be suitable for quantitating the enantiomeric composition of samples of naproxen based on the measurement of either peak areas or peak heights.

To confirm its validity, the proposed NMR method was used to assay a set of 10 synthetic mixtures of (*S*)-(+)- and (*R*)-(−)-naproxen made in the proportions shown in Table 1. The results of the assays were found to be in close agreement with the known weights of the individual enantiomers in the sample mixtures whether they were based on the integrals of the α-methyl or ester-methyl proton signals. At the same time, they were indicative of the good accuracy of the method. The mean ± SD recovery values were 99.39 ± 0.92% and 99.42 ± 0.68% of (*S*)-(+)-naproxen, depending on whether the quantitation is based on the α-methyl or ester-methyl proton signals, respectively.

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DRUG FORMULATIONS

Liquid Chromatographic and Spectral Analysis of the Stereoisomers of Dimethylaminorex

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The individual enantiomers of *cis*- and *trans*-3,4-dimethylaminorex were prepared by treating ephedrines or pseudoephedrines with cyanogen bromide. These compounds represent potential designer drug modifications of aminorex and 4-methylaminorex, which have appeared recently in the clandestine drug market. The UV spectra for these compounds are typical of phenethylamine-type compounds, and FTIR spectra allow for differentiation of *cis*- and *trans*-isomers. The mass spectra for the dimethylaminorex stereoisomers show characteristic fragments at *m/z* 57, 118, and 190. The *cis*- and *trans*-isomers were separated in a reversed-phase liquid chromatographic system on a C18 stationary phase, with the *cis*-isomer displaying the higher capacity factor.

The pharmacological properties and abuse potential of the various derivatives of 2-amino-5-aryl-2-oxazolines have received considerable attention in recent years (1–3). Early reports (4, 5) described the anorectic activity of a large series of these compounds as potential substitutes for the amphetamine-type anorectics. The original reports on 2-amino-5-phenyl-2-oxazoline (aminorex) described it as a potent anorectic with interesting central nervous system (CNS) stimulating properties (4). Its anorectic properties were initially examined in rats and showed it to be equipotent with *d*-amphetamine. Substitution of halogens, particularly fluorine and chlorine at the *para* position of the aromatic ring, yielded anorectic activity up to 4 times that of aminorex. Electron donating alkoxy groups on the phenyl ring reduced activity, as did complete aromatization of the heterocyclic system to yield the oxazole.

The methyl derivative of aminorex, 4-methylaminorex or 2-amino-4-methyl-5-phenyl-2-oxazoline, was shown (4) to possess considerable anorectic activity in rats, having a slightly higher ED_{50} than aminorex and activity comparable to racemic amphetamine. The addition of a methyl group at the 4-position introduces geometric (*cis-trans*) isomerism into these compounds. The anorectic properties of the racemic *cis*- and racemic *trans*-methylaminorex, as well as the (+)-*trans*-methylaminorex, were essentially equipotent.

The anorectic activity of various aminorex derivatives has been substantiated in humans, and early animal studies revealed that these compounds also possessed CNS stimulant and cardiovascular effects similar to those of amphetamine.

In recent years, racemic *cis*-methylaminorex has appeared among the growing number of designer drugs available on the clandestine market. This compound was also recently classified as a Schedule I substance. Recently, Glennon and Misenheimer (6) reported the stimulus-generalization properties of the 4 individual stereoisomers of 4-methylaminorex compared to (*S*)-(+)-amphetamine. These studies showed the *trans*-

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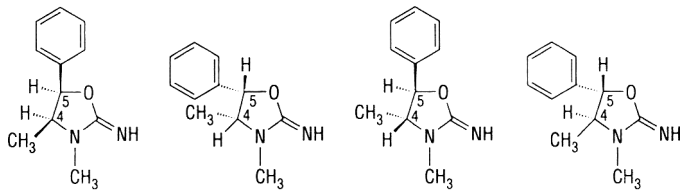


***cis*-(4*S*,5*R*); *cis*-(4*R*,5*S*); *trans*-(4*R*,5*R*); *trans*-(4*S*,5*S*)**

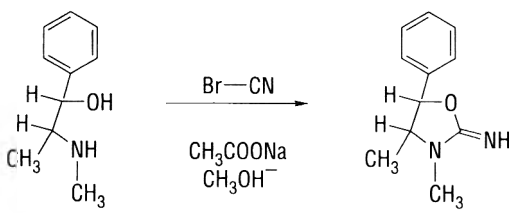
(4*S*,5*S*)-isomer to be more potent than either *cis*-isomer [the (4*S*,5*R*)- and (4*R*,5*S*)-isomers], which, in turn, were more potent than the *trans*-(4*R*,5*R*)-isomer. The more potent *trans*-(4*S*,5*S*)-isomer was found to be similar in potency to (*S*)-amphetamine. These stimulant and euphoriant effects, as well as blood pressure elevation, are likely the result of a sympathomimetic mechanism similar to amphetamine.

The stereoisomers of 4-methylaminorex have the potential to become significant problems in the clandestine drug market. These compounds can be prepared in a 1-step synthesis from readily available starting materials, norephedrine, norpseudoephedrine, and cyanogen bromide. Aminorex is prepared by an analogous synthesis from commercially available 2-amino-1-phenylethanol. Aminorex and 4-methylaminorex have already appeared on the clandestine street market (1, 3). The potential exists for the 3,4-dimethylaminorex isomers to appear in street samples as further designer modifications of the aminorex molecule. The dimethylaminorex isomers could be prepared via the same synthetic route described above from commercially available ephedrine and pseudoephedrine starting materials. In this study, we report the synthesis and analytical profiles of the 4 isomers of dimethylaminorex as "designer drug" analogues of aminorex and 4-methylaminorex.

Experimental

Instrumentation

The liquid chromatograph consisted of a Laboratory Data Control Constametric 3000 pump, 3100 spectromonitor UV detector operated at 220 nm, CI 4100 integrator, Rheodyne 7125 injector, and Waters Associates 30 cm × 3.9 mm μBondapak C18 column. Infrared spectra were recorded on a Perkin-Elmer Model 1710 Fourier transform infrared (FTIR) spectrophotometer. Ultraviolet spectra were recorded on a Shimadzu Instruments Model UV-160 spectrophotometer. Nuclear magnetic resonance spectra (^{1}H) were determined on a Varian EM-360 60 ^{1}H MHz spectrometer.



Scheme 1

Scheme 1. Synthesis of the stereoisomers of 3,4-dimethylaminorex.

Synthesis of cis- and trans-3,4-Dimethyl-5-phenyl-4,5-dihydro-2-amino-2-oxazolines (cis- and trans-3,4-Dimethylaminorex)

A solution of 1.6 g cyanogen bromide (15 mmol) in 10 mL methanol was added over a 10 min period to a cold (ice bath), stirred solution composed of 2.5 g of the appropriate ephedrine or pseudoephedrine (15 mmol) and 2.4 g sodium acetate (29 mmol) in 25 mL methanol. After the addition was complete, the mixture was stirred 1 h at room temperature and the solvent was evaporated under reduced pressure. The remaining oil was suspended in 25 mL water and made basic with 10% sodium hydroxide. The resulting oils were isolated by extraction with two 2 mL portions of chloroform and evaporation of the combined chloroform extracts. The product oils were crystallized from mixtures of carbon tetrachloride and ethyl acetate.

Liquid Chromatographic Procedures

The analytical column was 30 cm × 3.9 mm id packed with μBondapak C18 (Waters Associates). The analytical column was preceded by a 7 cm × 2.1 mm id guard column packed with CO:Pell ODS (Whatman). The derivatives were dissolved in LC-grade acetonitrile or methanol (1.0 mg/mL) and chromatographed with a mobile phase of pH 3.0 phosphate buffer and methanol (5 + 1). The phosphate buffer was prepared by dissolving 9.2 g monobasic sodium phosphate (NaH_2PO_4) in 1 L double-distilled water and adjusting the pH to 3.0 with H_3PO_4. The mobile phase flow rate was 1.5 mL/min, and the detector was operated at 0.2 AUFS. A 10 μL aliquot of sample solution was injected into the liquid chromatograph.

Results and Discussion

The analytical profiles for the isomers of methylaminorex (1) and aminorex (2) and its 4-phenyl regioisomer (7) have been reported previously. These compounds are all available through the same synthetic methodology: cyanogen bromide cyclization of the requisite phenethanolamine to yield the 2-oxazoline. Thus, it is reasonable that continued designer drug interest in this series will lead to the use of ephedrine or

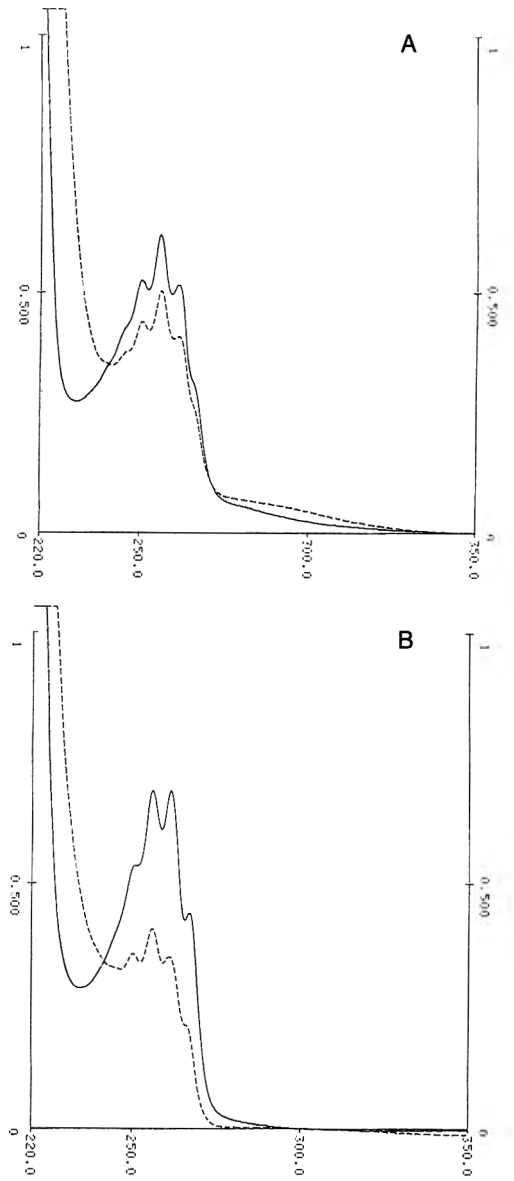


Figure 1. Ultraviolet absorption spectra of the isomers of 3,4-dimethylaminorex. A = *cis*-(*S,R*)-dimethylaminorex; B = *trans*-(*R,R*)-dimethylaminorex. Spectra represented by the solid line were determined in dilute sulfuric acid; spectra represented by the dashed line were determined in aqueous base.

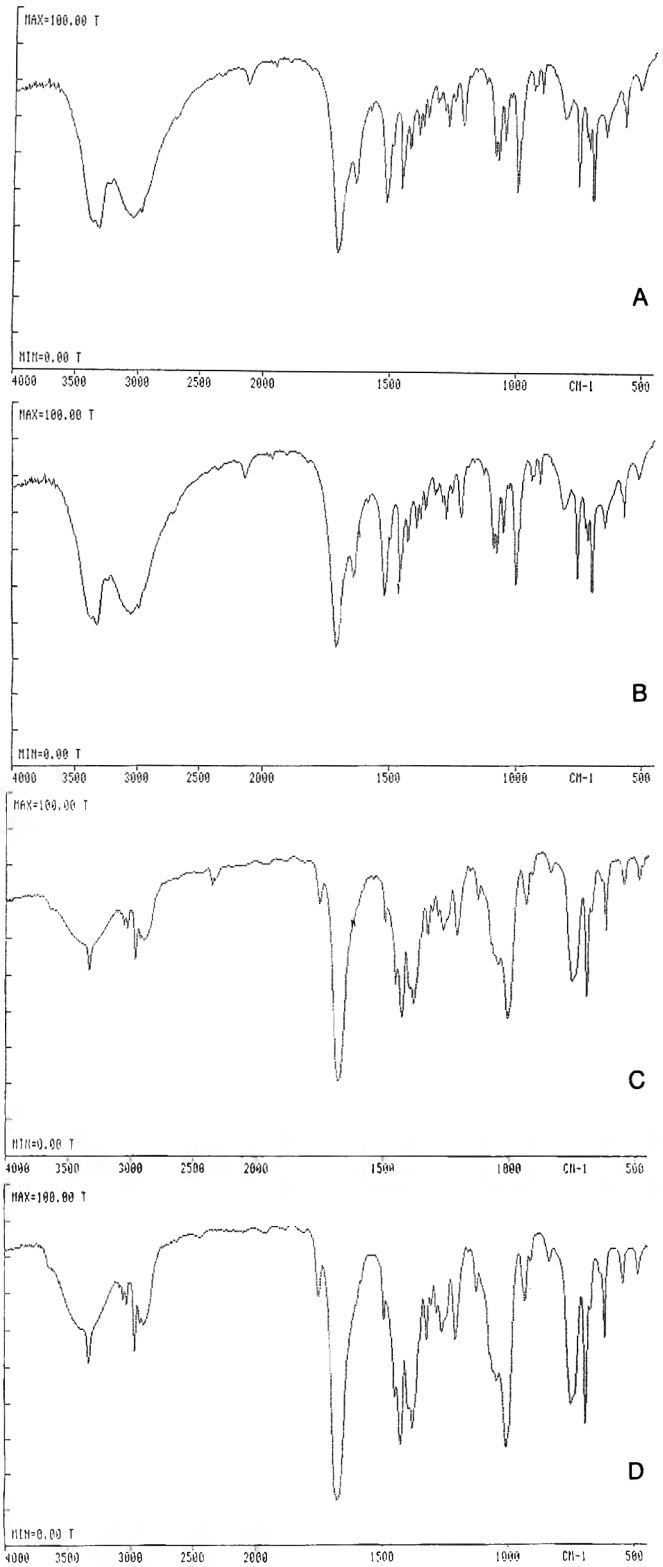


Figure 2. Infrared spectra of isomers of 3,4-dimethylaminorex. A = *cis*-(*S,R*)-dimethylaminorex, B = *cis*-(*R,S*)-dimethylaminorex, C = *trans*-(*R,R*)-dimethylaminorex, and D = *trans*-(*S,S*)-dimethylaminorex.

pseudoephedrine as the phenethanolamine moiety in the cyanogen bromide cyclization reaction. The stereoisomers of dimethylaminorex were synthesized by this procedure as shown in Scheme 1, and these compounds were found to exist in the exocyclic double bond form because of the additional methyl group on the amine moiety of the ethanolamine fragment. The melting points for the dimethylaminorex isomers were determined in open capillary tubes and were quite low compared to the isomers of methylaminorex. The *cis*-(*S,R*)-isomer melted at 94–97°C and the *cis*-(*R,S*)-isomer melted slightly lower, at 90–93°C. The *trans*-(*S,S*)-isomer melted at 38–42°C and the *trans*-(*R,R*)-isomer at 39–44°C. The low melting points of these compounds may contribute to the necessity for organic solvent extraction in the synthetic work-up procedure. The higher-

Table 1. Proton NMR data (ppm) for the dimethylaminorex stereoisomers[a]

Proton	*cis*-(4*S*,5*R*)	*cis*-(4*R*,5*S*)	*trans*-(4*S*,5*S*)	*trans*-(4*R*,5*R*)
2-N-H	4.72 s	4.65 s	4.25 s	4.25 s
3-CH_3	2.84 s	2.85 s	2.82 s	2.82 s
4-H	3.92 p (*J* = 7 Hz)	3.90 p (*J* = 7 Hz)	3.40 m	3.38 m
4-CH_3	0.71 d (*J* = 7 Hz)	0.73 d (*J* = 7 Hz)	1.25 d (*J* = 7 Hz)	1.22 d (*J* = 7 Hz)
5-H	5.49 d (*J* = 8 Hz)	5.47 d (*J* = 8 Hz)	4.82 d (*J* = 9 Hz)	4.80 d (*J* = 9 Hz)
5-Ar-H	7.35 s	7.33 s	7.38 s	7.35 s

[a] All NMR spectra were determined in $CDCl_3$ with tetramethylsilane as an internal standard. Signal multiplicities are designated as follows: s = singlet, d = doublet, m = multiplet, and p = pentet.

melting methylaminorex isomers precipitate upon addition of base to the reaction mixture. The individual isomers of methylaminorex melt at temperatures above 177°C (1).

The ultraviolet absorption spectra of *cis*-(*S*,*R*)- and *trans*-(*R*,*R*,)-dimethylaminorex are shown in Figure 1. These spectra show the general absorption bands for phenethylamines in the 240–270 nm range. In Figure 1A, the spectrum for the *cis*-isomer shows 1 major absorption band with 2 slightly less intense bands in both acid and base solution. The spectrum for the *trans*-isomer in Figure 1B shows 2 bands of almost equal intensity in acid with a single major absorption of significantly lower intensity in base.

The infrared absorption spectra of the free base form of each isomer of dimethylaminorex are shown in Figure 2. These spectra were obtained from KBr disks on a Fourier transform infrared spectrophotometer. Because these compounds were synthesized as the individual stereoisomers, no spectra were obtained for racemic *cis*- or racemic *trans*-dimethylaminorex. The infrared spectra for the individual enantiomers of *cis*- can clearly be distinguished from the enantiomers of *trans*-dimethylaminorex. The spectra for the 2 *trans*-isomers (4*R*,5*R* and 4*S*,5*S*) appear identical in all respects, but slight differences in relative intensity exist between the individual stereoisomers of *cis*-dimethylaminorex.

The ^1H NMR data for the *cis*- and *trans*-dimethylaminorex isomers are shown in Table 1. The imino proton appears at a slightly higher field in the *trans*-isomers, while the *N*-methyl group does not appear to be influenced by the geometry of the 4,5-substituents. The methyl group at C-4 is upfield in the *cis*-isomer, appearing as a doublet centered at 0.7 ppm, whereas this signal in the *trans*-isomers occurs as a doublet at 1.2 ppm. This same trend was observed for the C-4 methyl group in the *cis*- and *trans*-isomers of 4-methylaminorex (1). The proton at C-4 occurs as a pentet centered at 3.9 ppm for *cis*-dimethylaminorex and as a multiplet centered at 3.4 ppm for the *trans*-isomer. The proton at C-5 occurs as a doublet in the spectrum of both isomers, with the signal for the *cis*-isomer slightly downfield compared to the *trans*-dimethylaminorex.

The mass spectra for these dimethylaminorex isomers are identical and an example spectrum (EI) is shown in Figure 3. The molecular ion is presented at *m/z* 190 as well as a peak for *m/z* 175 (M-15) most likely resulting from the loss of a methyl group. Because 4-methylaminorex shows an analogous loss of 15 mass units, the M-15 peak likely arises from the loss of the C-4 methyl group. The *m/z* 118 ion in the dimethylaminorex

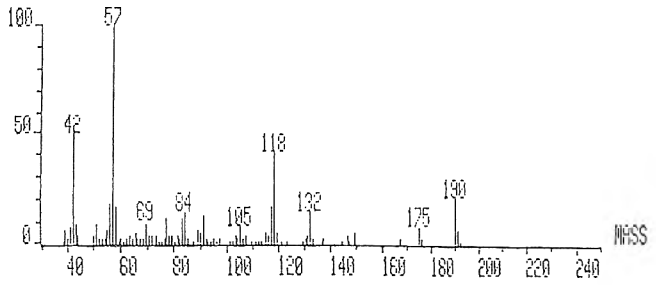


Figure 3. Mass spectrum of *cis*-(*S*,*R*)-dimethylaminorex.

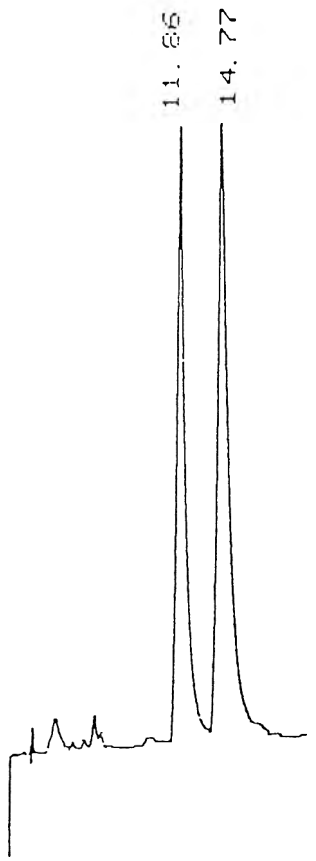
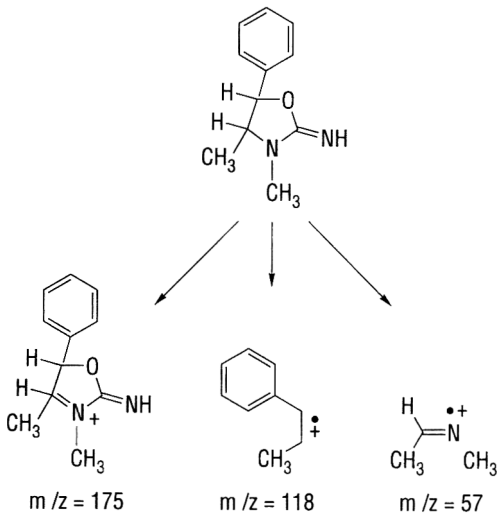


Figure 4. Reversed-phase liquid chromatographic separation of *trans*-dimethylaminorex (11.86 min) and *cis*-dimethylaminorex (14.77 min).



Scheme 2

Scheme 2. Mass spectral fragmentation scheme for the stereoisomers of 3,4-dimethylaminorex.

isomers is not observed in the spectrum of methylaminorex and likely is the phenylpropane skeleton, C_9H_{10} (Scheme 2). The base peak in the dimethylaminorex spectra occurs at *m/z* 57 and likely results from a retro-Diels-Alder-type fragmentation to yield C_3H_7N by splitting out the C–N, rings positions 4 and 3, respectively, and the accompanying methyl substituents. According to data from Klein et al. (1), the most abundant ion at *m/z* 43 in the EI-MS of methylaminorex has the elemental composition C_2H_5N. This ion possibly originates from the 2-imino tautomer by the loss of the N-3 and C-4 with its methyl substituent (CH_3–CH=NH). The analogous reaction with dimethylaminorex, which is locked in the 2-imino tautomeric form, would yield the *m/z* 57 ion due to the additional methyl group present on N-3.

The liquid chromatographic separation of the *cis*- and *trans*-isomers of dimethylaminorex is shown in Figure 4. This separation was achieved in the reversed-phase mode with a C18 stationary phase and a mobile phase of pH 3 phosphate buffer and methanol (5 + 1). The peak eluting first corresponds to the *trans*-isomer (11.86 min) and the *cis*-isomer elutes approximately 3 min later (14.77 min). The chromatographic system used for this separation is commonly used in our laboratory for the analysis of other basic drugs. However, further refinements of the system will be necessary to adequately resolve the isomers of both dimethylaminorex and methylaminorex in a single isocratic system.

In summary, the 3,4-dimethylaminorex isomers can be prepared from cyanogen bromide cyclization of ephedrine or pseudoephedrine. These compounds represent potential designer drug modifications of the 2-amino-5-phenyl-2-oxazoline (aminorex) system. These dimethyl derivatives contain the exocyclic imino (C=NH) double bond due to the additional *N*-methyl substituent at the 3-position of the oxazoline ring. These compounds are low-melting solids that show UV absorption properties characteristic of phenethylamine-type compounds. The *cis*- and *trans*-isomers can be separated by liquid chromatography with a reversed-phase system, and the *trans*-isomer displays the lower capacity factor. The *cis*- and *trans*-isomers can be differentiated on the basis of their infrared absorption spectra, and the EI-MS for all isomers show characteristic fragments at *m/z* 57 (base peak), 118, and 190 (molecular ion).

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DRUG RESIDUES IN ANIMAL TISSUES

Matrix Solid-Phase Dispersion Isolation and Liquid Chromatographic Determination of Oxolinic Acid in Channel Catfish (*Ictalurus punctatus*) Muscle Tissue

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Matrix solid-phase dispersion isolation and liquid chromatographic techniques were developed to quantify oxolinic acid (OA) and OA-related metabolites in channel catfish (*Ictalurus punctatus*) muscle tissue and bile. Mean percent recovery, correlation coefficient, and inter- and intra-assay variabilities were 82.8 ±15.0%, 0.996 ±0.004, 12.5 ±8.9%, and 1.22%, respectively, for OA isolated from fortified muscle tissue. Using the methodologies described in the current study, incurred OA in muscle tissue and 4 OA-related metabolites were isolated in the bile of dosed catfish.

Management of an economically successful aquaculture facility requires that fish number be maintained at high production levels. Fish health under such conditions is often compromised by deteriorating water quality and ensuing bacterial epizootics. Antibiotic therapy, when used, may result in drug residues that are of significance to drug efficacy and health considerations for the human consumer.

Oxolinic acid (OA), 5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-g]quinoline-7-carboxylic acid, is a quinolone antibiotic efficacious against gram-negative bacterial fish pathogens (1, 2). Characterization of OA distribution, metabolism, and retention in fish requires methods for quantitation in biological matrixes. Many of the current isolation techniques for OA in fish tissue are time-consuming, involve multiple steps, and require large solvent volumes (3–7). New strategies for drug isolation that use blending of a sample matrix with an octadecylsilyl-derivatized solid support were developed (8–18). These isolation techniques, known as matrix solid-phase dispersion (MSPD), were successfully used to isolate 2 antibiotics from fortified fish muscle tissue, oxytetracycline (OTC) (17) and sulfadimethoxine (SDM) (18). Currently, few studies are available that demonstrate the utility of this method with incurred residues of any compound.

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The intent of the present study was to develop MSPD and liquid chromatography (LC) techniques suitable for use with OA and its respective metabolites, and to determine percent recovery, standard curve correlation coefficients, and inter- and intra-assay variabilities of OA from fortified fish tissue.

Experimental

Reagents and Apparatus

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

(b) *Water.*—For LC analysis, double deionized and passed through Modulab Polisher I water purification system (Continental Water Systems Corporation, San Antonio, TX 78238).

(c) *Oxolinic acid (OA).*—Argent Chemical Laboratories, Inc., Redmond, WA .

(d) *Piromidic acid (PA).*—Sigma Chemical Co., St. Louis, MO 63178.

(e) *Sodium hydroxide.*—EM Science, Cherry Hill, NJ.

(f) *Glacial acetic acid (GAA).*—Mallinckrodt, Inc., Paris, KY.

(g) *Column material.*—Bulk C18, 40 μm, 18% load, end-capped (Analytichem International, Harbor City, CA), cleaned by making a column (50 mL syringe barrel) of the bulk C18 material (22 g) and washing sequentially with 2 column volumes each of hexane, methylene chloride (DCM), and methanol. C18 material was vacuum aspirated until dry and stored in glass.

(h) *Stock oxolinic acid solution.*—2 mg/mL. OA was partially dissolved in methanol, and 2 mL 1.0N NaOH was added for complete OA dissolution. Methanol was added to bring the final volume to 1 L.

(i) *Stock piromidic acid solution.*—0.16 mg/mL. PA was partially dissolved in methanol, and 100 μL 1.0N NaOH was added to bring PA completely into solution. Methanol was added to bring the final volume to 100 mL.

(j) *Stock solutions for standard curves.*—Prepared by adding pure methanol to 37.5 (750 μg/mL), 25.0 (500 μg/mL), 12.5 (250 μg/mL), 2.5 (50 μg/mL), 1.25 (25 μg/mL), and 0.25 mL (5 μg/mL) stock OA to make a 100 mL final volume. A 780 μL volume of 0.05M GAA–100% MeOH (1 + 1), 10 μL

OA standard, and 10 μL PA internal standard were mixed in an LC vial to serve as standards.

(k) *Sample extraction columns.*—Syringe barrels (12 mL); washed in hot soapy water, rinsed with experimental water, and air-dried before use.

Sample Preparation and Extraction Procedure

Channel catfish (*Ictalurus punctatus*) used in this study were obtained from the Ben Hur Aquaculture Research Unit, Louisiana State University, Baton Rouge, LA. Fish were maintained at least 3 weeks before experimentation under flow-through conditions in carbon-filtered, pH, and hardness adjusted water (pH 8.3, hardness 21 mg/L as $CaCO_3$, alkalinity 171 mg/L as $CaCO_3$, and 24°C).

Skinned catfish muscle tissue excised from the epaxial group was used in the fortification studies, which evaluated percent recoveries, standard curve correlation coefficients, and inter- and intra-assay variabilities. Muscle tissues (0.5 g) were individually fortified with standard OA (10 μL, 5.0–750 μg/mL) and internal standard (10 μL, 0.16 μg/μL). Once fortified, tissues were undisturbed for 5 min. Blank tissue controls (0.0 μg/g) were prepared by the addition of 10 μL 100% methanol and 10 μL PA internal standard.

Incurred residues in muscle and bile were derived from a catfish (240 g) administered 1.2 mg ^{3}H-OA (previously purified to 99.8% by thin-layer chromatography and LC verified) with a specific activity of 0.016 μCi/mM. Catfish were dosed via gavage administration.

Extraction procedures were essentially the same for muscle and bile generated in both phases of the study. The only differences resulted from the degree of mixing required for the 2 biological matrixes. Muscle or bile samples (40 μL) were individually placed onto 2.0 g C18 in a glass mortar. Tissue samples were then blended into C18 with a glass pestle until the sample mixture appeared homogeneously dispersed over the C18. The C18/tissue mixture was then quantitatively transferred to a 12 mL syringe plugged with a filter paper disc (Whatman No. 1, 15 mm, Whatman International Ltd, Maldstone, England). Column contents were compressed to 4.0 mL using the syringe plunger (rubber end and plastic tip removed). A 100 μL pipet tip was fitted to the syringe barrel outlet to retard solvent flow through the column.

Column containing C18/matrix was first washed with 8 mL hexane. Once flow ceased, excess hexane was removed from the column by gently applying positive pressure from a pipet bulb. The hexane fraction was not found to contain OA, so it was discarded. OA, internal standard, and metabolites (when applicable) were eluted from the column by sequentially washing with 8 mL each of acetonitrile and methanol. Acetonitrile and methanol fractions were collected in a single 18 × 150 mm disposable borosilicate glass culture tube. The eluant was concentrated to dryness under nitrogen gas with heat (45°C) maintained by a water bath. During drying, tubes were periodically rinsed with methanol to remove any residue from glass walls. Samples were either analyzed immediately or stored at −14°C under nitrogen gas. Before LC analysis, dried residue was reconstituted in 1600 μL 0.05M GAA–methanol (1 + 1), vortexed 30 s, and filtered through a 0.22 μm nylon 13 mm polypropylene encased syringe filter (Alltech Associates Inc., Deerfield, IL 60015). An 800 μL aliquot of sample was analyzed by LC.

Liquid Chromatographic Analysis

LC analysis of fortified muscle samples, OA standards, and incurred residue were conducted using the following chromatographic system: Waters M-6000A chromatography pumps (Waters Associates Inc., Milford, MA 01757); Micrometrics 728 Autosampler and Model 732 Electronic Actuator (Micrometrics Instrument Corp., Norcross, GA 30093); Waters Guard-Pak guard column, 500 μL injection loop; Alltech Versapack C18 LC column, 10 μm, 250 × 4.1 mm id; Spectroflow 783 UV detector (Kratos Analytical, Ramsey, NJ 07446) set at 260 nm; and a Hewlett-Packard 3392A integrator (Hewlett-Packard Co., Avondale, PA). Column temperature was maintained at 40°C for all determinations. Samples were analyzed under gradient conditions at 1 mL/min using 100% methanol (Solvent A) and 0.05M GAA (Solvent B). Conditions were initially 0% Solvent A and 100% Solvent B for 5 min, then changed at 3%/min for 20 min to 60% Solvent A, 40% Solvent B. Conditions were held constant for 10 min, then Solvent A content was increased to 100% over 10 min. The column was returned to initial chromatographic conditions over the next 10 min and maintained there for 10 min to allow for adequate reequilibration between injections. Under these chromatographic conditions, OA and PA eluted at 26.2 and 31.1 min, respectively.

In metabolite determinations of ^{3}H-OA incurred residues, all eluant fractions associated with peaks from the chromatographic run were collected. Fractions were counted for radioactivity using a Packard Tricarb Liquid Scintillation Counter, Model 4640 (Packard Instrument Co., Downers Grove, IL). Fractions containing radioactivity significantly above background were defined as those containing OA-derived metabolites.

Data Analysis

Peak area ratio (PAR) curves of OA standards and samples were determined by plotting integration areas of the generated OA peaks vs PA internal standard peaks. A comparison of extracted fortified sample PARs to the PARs of pure standards under identical chromatographic conditions gave percent recoveries. The means and standard deviations (SD) were determined for 5 replicates of each concentration. Coefficient of variation (CV) was then obtained by dividing the SD by its respective mean and multiplying this value by 100. Interassay variability was determined by averaging CVs (±SD) over all concentrations. Intra-assay variability was estimated as the CV of the mean for 5 replicates of the same sample. Intra-assay variability is an estimation of the variation associated with the analytical instrumentation.

Results

OA percent recoveries ranged from 63.0 ±19.8 to 100.2 ±3.9, and percent recovery increased with OA concentration

Table 1. Concentration range, mean (±1 SD) percent recoveries, standard curve correlation coefficient, and inter- and intra-assay variabilities of oxolinic acid isolated from oxolinic acid-fortified channel catfish (*Ictalurus punctatus*) muscle tissue

Oxolinic acid concn, μg/g tissue	Mean (±1 SD) rec., %
0.1	63.0 ± 19.8
0.5	65.8 ± 5.7
1.0	90.5 ± 7.5
5.0	85.9 ± 8.3
10.0	91.7 ± 12.1
15.0	100.2 ± 3.9
Mean recovery	82.8 ± 15.0
Correlation coefficient	0.996 ± 0.004
Interassay variability, %	12.5 ± 8.9
Intra-assay variability, %	1.22

(r = 0.789). PARs were linear from 0.1 to 15.0 μg/g, and the correlation coefficient was 0.996 ±0.004. Inter- and intra-assay variations were minimal at 12.5 ±8.9 and 1.22%, respectively. Other response variables investigated in this study are presented in Table 1. Figure 1 shows chromatograms for extracted blanks, fortified muscle tissue, and incurred residue in muscle.

The MSPD isolation and subsequent LC analysis of bile using the described methods resulted in a separation of 4 OA-related metabolites (Peaks 1–4) and parent OA (Peak 5), as seen in Figure 2.

Discussion

MSPD involves the dispersion of a sample matrix over a large surface area by using both mechanical and hydrophobic forces. Pressure applied by the tissue grinding process causes lipids and outer-membrane structures to interact with the non-polar C18 support, which results in a disruption of the tissue matrix. Compounds of interest are then preferentially eluted from the C18/tissue matrix by a selection of solvents with polarity similar to that of the analyte (8–18).

The advantages of MSPD isolation over traditional extraction techniques have been thoroughly discussed (8–17). The present study demonstrates that many of the same advantages also exist for the extraction of OA from catfish tissue. Current methods for the isolation of OA from fish tissue required 20–61 min, solvent volumes of 60–560 mL, sample sizes of 5.0–10.0 g, and 7–10 procedural steps (2–6). In contrast, MSPD isolation of OA requires only 15–25 min for complete tissue processing and column elution, 24 mL elution solvents, 0.5 g tissue, and no more than 4–5 procedural steps.

Results of the current study indicate that MSPD isolation of OA from fish tissue yields a mean percent recovery of 83% over a range of concentrations from 0.1 to 15.0 μg/g. Other studies that investigated extraction procedures for OA reported percent recoveries ranging from 77 to 105% (2–6). In these studies, percent recoveries were derived using OA concentrations of 1 or 2 μg/g (2, 5–7). Using MSPD, OA recovery within a comparable range of concentrations was greater than 90.0%.

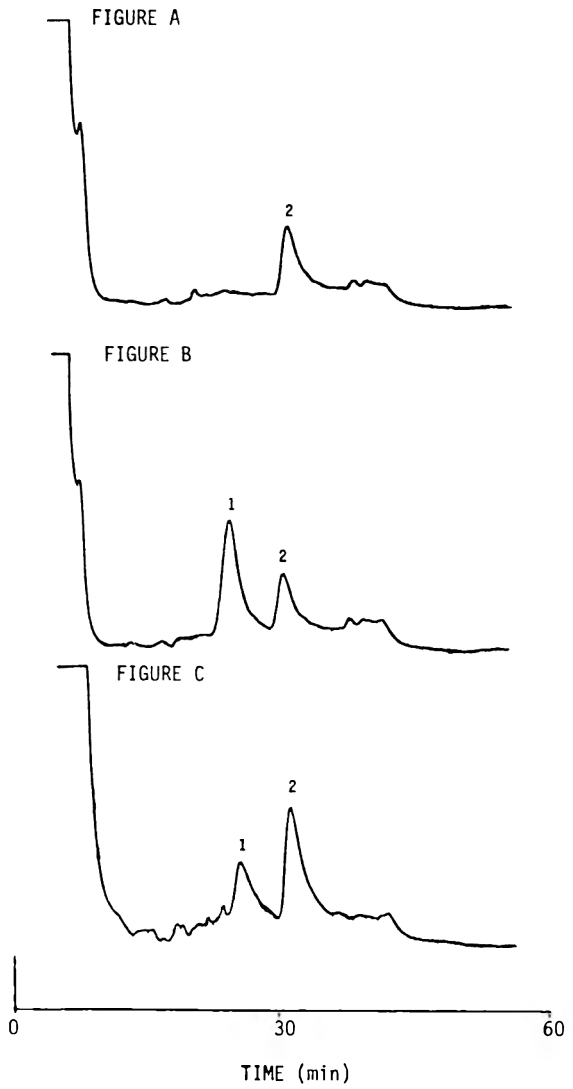

Figure 1. Representative liquid chromatograms of (A) MSPD-extracted muscle tissue blank containing 3.2 μg/g piromidic acid (Peak 2), (B) 5.0 μg/g oxolinic acid (Peak 1) and 3.2 μg/g piromidic acid (Peak 2) isolated from fortified channel catfish muscle tissue, and (C) incurred oxolinic acid (Peak 1) and piromidic acid (Peak 2) isolated from muscle tissue of dosed channel catfish.

The current study indicates MSPD coupled with LC can be used to quantify OA either as incurred residue or from fortified muscle tissue. The isolation of incurred residues can provide important metabolic information for ascertaining risk and efficacy. MSPD was also used to isolate OTC and SDM (17, 18). Even though percent recoveries of these compounds from fortified tissue were 82.0 and 101.7% for OTC (17) and SDM (18), respectively, these studies did not attempt to demonstrate the use of MSPD to extract the incurred antibiotic residues.

Initially, a slow gradient (3%/min) was used to determine the presence of ^{3}H-OA-related metabolites in tissue. This gra-

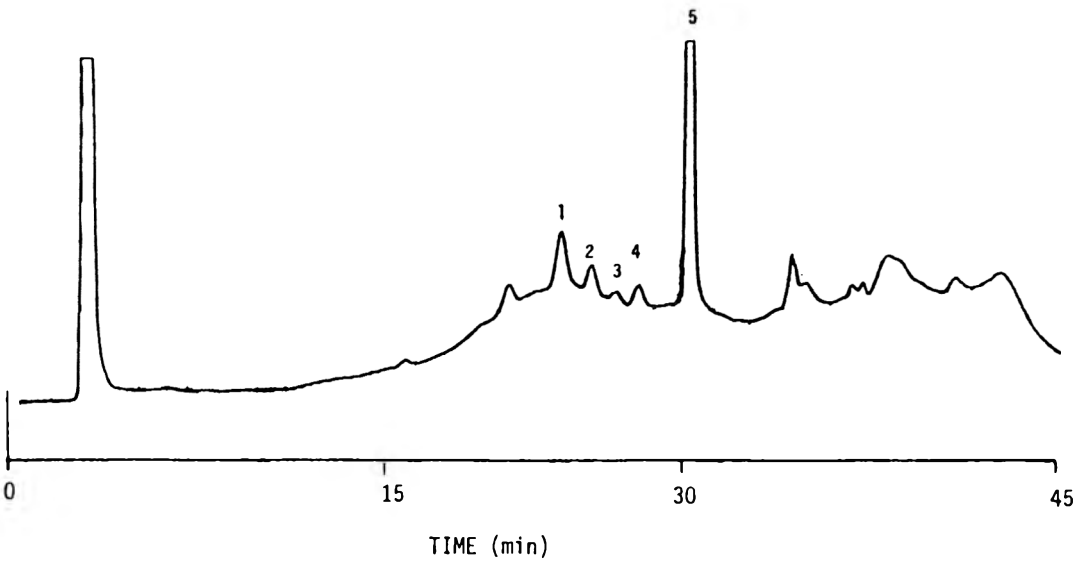

Figure 2. Liquid chromatogram of 40 μL channel catfish bile sample extracted using MSPD. Peaks 1–5 were those identified as containing radioactivity.

dient proved adequate for the muscle tissue because metabolites were not detected and the peak for OA was well-resolved; however, to completely resolve all metabolites in the bile, the gradient has to be reduced to 2%/min over 35 min. Under these chromatographic conditions, 5 radioactive peaks were fully resolved. Before the current study, OA metabolites had not been isolated from channel catfish.

To optimize chromatographic conditions and isolation methodologies for parent OA, LC run time may be shortened by stepping the gradient to 6%/min, which results in an OA retention time of about 16 min. Increasing the gradient to this rate will provide good peak resolution of both parent OA and internal standard; however, metabolites will elute with the solvent front.

Using the chromatographic system of the current study, the proven limit of detection for OA was 0.05 μg/g. Limits of detection for OA in fish tissue ranging from 5.0 to 100 μg/g were reported using other isolation techniques and chromatographic systems (19–21). Sensitivity can be increased for the parameters investigated in this study by increasing injection volume, extracting greater amounts of tissue, or by further concentrating the sample before LC analysis.

The current research presents an MSPD technique for the isolation of OA from fortified channel catfish muscle tissue and incurred residue. The method yields an average percent recovery of 83.0%. Additionally, the LC conditions described in the present study can be used effectively to resolve both parent OA and metabolite residue from catfish muscle tissue and bile.

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DRUGS IN FEEDS

Liquid Chromatographic Determination of Gentian Violet in Poultry Feed

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A liquid chromatographic (LC) method is presented for the determination of gentian violet (GV) in poultry feed (turkey/chicken) at the therapeutic feeding level of 4–8 ppm. GV is extracted from feed with acidified methanol, an aliquot of the supernatant is diluted with mobile phase, and the solution is filtered. LC analysis is performed by isocratic elution with a buffered mobile phase on an Alltech CN (cyano) column with amperometric electrochemical detection (ED) at +1.000 V or detection in the visible absorbance mode at 588 nm. The overall average recovery of GV from chicken feed spiked at 2.5, 5, and 10 ppm was 103% (standard deviation = 6.6; coefficient of variation = 6.4%) by LC/ED analysis. Data for recovery of GV from chicken and turkey feeds, fortified with 1% GV premix at feeding levels of 4 and 8 ppm, are presented and discussed. Data for the 2 detection techniques are compared.

Typical approaches for determining gentian violet (GV) have involved the use of spectrophotometry, thin-layer chromatography, and liquid chromatography (LC) with measurement of absorbance at 588 nm (VIS) (1–7). In 1980, Rushing and Bowman (8) reported an LC/VIS method for determining GV in animal feed, human urine, and wastewater. The analysis of feed by that method involves extraction with methanol–1N HCl (99 + 1), followed by isolation and cleanup on a Sephadex column with benzene–methanol before LC/VIS determination. In 1987, Cambridge Products, Ltd, documented a method for the determination of GV in premixes and fortified animal feeds (9). The method was based on the work of Rushing and Bowman in which they used a toluene–methanol solvent mixture in the Sephadex column. Martinez and Shimoda (10) modified the Rushing and Bowman procedure by substituting the less hazardous solvent combination of methanol and water in the Sephadex column step. Although these procedures appear to be satisfactory, the use of the Sephadex column adds a long and labor-intensive step to the analysis.

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In a recent publication, LC conditions were reported for the electrochemical detection (ED) of GV (11). The method presented here incorporates the use of ED in the determination of GV in poultry feed at a feeding level of 4–8 ppm. This method eliminates the use of the Sephadex column and the evaporation steps. In addition to decreasing analytical time and increasing sample throughput, it reduces the volume of hazardous waste generated. This procedure is also very well suited for detection in the visible range if the UV/VIS spectrophotometric detector passes a simple sensitivity test.

METHOD

Apparatus

(a) *Syringes.*—Microliter, 25 μL (No. 802, Hamilton Co., Reno, NV 89502); glass, 5 mL (Becton Dickinson Microbiology Systems, Cockeysville, MD 21030).

(b) *Pasteur pipet.*—Disposable, 5.75 in. (14.6 cm).

(c) *Centrifuge bottle.*—500 mL with glass stopper, T 29/26 (K-322000-0022, Kontes Co., Vineland, NJ 08360).

(d) *Centrifuge tube.*—15 mL, graduated, with glass stopper, No. 13 (Cat. No. 45153-A, Kimble Division, Owens-Illinois, Inc., Toledo, OH 43666).

(e) *Feed grinder.*—Microjet 10-ZM 1, equipped with 1 mm screen (Micro Materials Corp., Westbury, NY 11590).

(f) *Mechanical shaker.*—3D floor shaker, Model VS55202 with variable movement control (Glas-Col Apparatus Co., Terre Haute, IN 47802).

(g) *Liquid chromatograph.*—Waters Model 6000-A LC pump and Model U6K universal LC injector (Waters Associates, Milford, MA 01757). Operating conditions: chart speed, 0.25 cm/min; mobile phase flow, 1.0 mL/min; column temperature, ambient; column pressure, 2500 psi; injection volume, 20 μL.

(h) *Detectors.—Electrochemical detector.*—BAS Model LC-4B single electrode detector (Bioanalytical Systems, Inc., Purdue Industrial Research Park, West Lafayette, IN 47906) with glassy carbon electrode, Ag/AgCl reference; working potential +1.000 V; current range, 5 or 10 nA FSD. *UV/VIS detector.*—Shimadzu ultraviolet-visible spectrophotometric detector, SPD-6AV module for LC (Shimadzu Corp., Analytical Instruments Division, Kyoto, Japan); cell volume, 8 μL;

Table 1. Recovery of GV from spiked control chicken feed (5% tallow) by LC/ED

Detn	Added, ppm	Rec., ppm	Rec., %
1	2.53	2.55	101
2	2.53	2.73	108
3	2.53	2.60	103
4	2.53	2.75	109
5	2.53	2.78	110
6	5.06	5.46	108
7	5.06	5.56	110
8	5.06	4.99	98.6
9	5.06	5.35	106
10	5.06	5.30	105
11	10.1	9.98	98.8
12	10.1	10.5	104
13	10.1	9.88	97.8
14	10.1	8.58	85.0
15	10.1	9.88	97.8
$\bar{X}$			103
SD			6.6
CV, %			6.4

Table 2. LC/ED determination of GV in premix[a]

Weight of premix analyzed, g[b]	GV found, % of label
1.0042	99.6
0.9948	92.0
1.0022	101
1.0052	96.9
1.0033	103
$\bar{X}$	98.5
SD	4.3
CV, %	4.3

[a] Used by CVM, Beltsville, MD, to prepare fortified feeds.
[b] Premix dilution factor in these analyses = 1000.

light source, tungsten iodide (WI) lamp (370–700 nm); absorbance range, 0.005 AUFS.

(i) *LC column.*—Alltech, CN (cyano), 5 μm particle size, 250 × 4.6 mm id (Cat. No. 605CN (new Cat. No. 60138), Alltech Associates/Applied Science, Deerfield, IL 60015), or equivalent.

(j) *Recorder.*—Dual channel SE-120 strip chart recorder set at 10 mV (BBC-Metrawatt/Goerz, Broomfield, CO 80020).

(k) *Filter.*—Millipore, disposable, 5 μm polytetrafluoroethylene (PTFE) membrane (Cat. No. SLSR 025 NB, Millipore Corp., Bedford, MA 01730).

Reagents

(a) *Solvents.*—Distilled-in-glass, pesticide grade methanol and UV spectro-grade acetonitrile (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442), or equivalent.

(b) *Water.*—LC grade (Fisher Scientific, Fair Lawn, NJ 07410) or deionized, glass-distilled.

(c) *Acetic acid.*—ACS grade, glacial, aldehyde-free.

(d) *Sodium acetate.*—ACS grade, anhydrous.

(e) *Extracting solution.*—Methanol–1N HCl (99 + 1).

(f) *Acetate buffer.*—Prepare by adjusting 0.1M sodium acetate solution (8.2 g sodium acetate/1000 mL water) to pH 4.5 with acetic acid (ca 8 mL). Use to prepare mobile phase, (g).

(g) *Mobile phase.*—Acetonitrile–acetate buffer (60 + 40).

(h) *Reference standards.—Gentian violet.*—USP, crystal violet 96%. *Stock standard (100 μg/mL).*—Accurately weigh 10.0 mg reference standard into 100 mL volumetric flask, dilute to volume with methanol, and mix. *Intermediate standard (1.0 μg/mL).*—Pipet 1.0 mL stock solution into 100 mL volumetric flask, dilute to volume with methanol, and mix. *LC/ED working standard (0.1 μg/mL).*—Pipet 1.0 mL intermediate standard into 15 mL centrifuge tube, dilute to 10.0 mL with mobile phase, and mix. Prepare weekly or as needed.

Sample Preparation

Grind feed to pass 1 mm sieve. Mix ground feed and store in quart glass jar. Keep in cool, dry place. (*See* Results and Discussion.)

Extraction

Accurately weigh 20 g ground feed into 500 mL centrifuge bottle. Add 200.0 mL methanol–1N HCl (99 + 1) and shake 1 h on mechanical shaker with vigorous motion. Let stand overnight.

Cleanup

Dilute appropriate aliquot of supernatant into 15 mL centrifuge tube, according to the following schedule (GV level (ppm), aliquot (mL)): 10 ppm, 1.00 mL; 5 ppm, 2.00 mL; and 2.5 ppm, 4 mL; if GV level is unknown, use 4 mL aliquot. Dilute aliquot to 10.0 mL with mobile phase and mix. Transfer solution to syringe fitted with PTFE filter, (k). Filter solution into 25 mL g/s Erlenmeyer flask.

Liquid Chromatography

Inject 20 μL filtered solution into liquid chromatograph. Bracket injections for each set of filtered solutions with 20 μL injections of GV working standard (0.1 μg/mL). Calculate concentration (ppm) of GV in feed as follows:

$$ppm = (P/S) \times (C/W) \times (D)$$

where P = peak height (mm) obtained from injection of filtered solution, S = average peak height (mm) obtained from injection of standard, C = concentration (μg/mL) of injected standard, D = final volume (mL) of filtered solution, and W = initial weight (g) of feed sample taken for analysis.

Results and Discussion

In our laboratory, the electrochemical detector has proved to be a very effective tool for selective residue analyses because of its sensitivity and selectivity. Our intent in this project was

Table 3. Recovery of GV from fortified feeds by LC/ED

Feed[a]	GV added, ppm[b]	Analyzed as received: GV found, ppm[c]	Rec., %	CV, %	Analyzed after grinding[d]: GV found, ppm[c]	Rec., %	CV, %
A–11	4	4.1[e]	102	10.4	—	—	—
A–12	8	9.6[e]	120	11.7	7.9	99	4.2
A–13	0	0	—	—	0	—	—
B–11	8	6.6	82	33.5	7.6	95	8.5
B–12	0	0	—	—	0	—	—
B–13	4	3.4	85	29.6	—	—	—
A–21	8	8.0	100	13.5	7.1	89	14.1
A–22	0	0	—	—	0	—	—
A–23	4	4.4	110	26.3	3.0	75	10.4
B–21	0	0	—	—	0	—	—
B–22	4	3.7	92	26.4	3.3	82	11.2
B–23	8	7.1	89	9.9	7.5	94	16.1
$\bar{X}$			98			89	
SD			13			9.0	
CV, %			13.3			10.1	

[a] A = chicken feed (5% tallow); B = turkey feed (1% tallow).
[b] Fortified feeds received from CVM, Beltsville, MD.
[c] Average of 8 determinations except where noted.
[d] To pass 1 mm sieve.
[e] Average of 6 determinations.

to simplify the determination of gentian violet in poultry feeds. In addition, we wanted to minimize hazardous waste generation. The method described is easier and more solvent-efficient than previously published methods (8–10).

Control and fortified feeds were furnished by the U.S. Food and Drug Administration's Center for Veterinary Medicine (CVM) in Beltsville, MD. The first shipment of feeds consisted of 2 types: chicken and turkey containing 5 and 1% tallow, respectively. Each type of fortified feed contained GV at 4 and 8 ppm. CVM later supplied a second shipment of fortified feeds, consisting of chick basal diet containing GV at 0, 2, 4, 6, 8, and 10 ppm.

The extraction method used was that of Rushing and Bowman (8), who determined that a methanol–1N HCl (99 + 1) extraction solution produced the cleanest extract and consequently good recoveries of GV. Mechanically shaking the extraction mixture for 1 h resulted in an 86% recovery of GV. The control chicken feed furnished by CVM was spiked and used for recovery analyses. Table 1 shows the overall recovery of GV added at ca 2.5, 5, and 10 ppm to control chicken feed (5% tallow). Recoveries for the individual levels were 107% (2.5 ppm, standard deviation (SD) = 4, coefficient of variation (CV) = 3.7%); 106% (5 ppm, SD = 4, CV = 3.8%); and 97% (10 ppm, SD = 7, CV = 7.2%). The premix used by CVM to prepare the fortified feeds was also analyzed and found to contain GV at 98.5% of the label declaration (Table 2).

Data previously obtained indicated that the developed method is capable of producing reasonable CVs (Table 1). Sample uniformity and extraction efficiency appear to be the 2 major determining factors of this method. The original analysis, which was performed on well-mixed "as received" fortified feed, resulted in large CVs. Preparing feeds that are homogeneous is very difficult for GV-medicated feeds. Although grinding the feed improves the CVs, the heat and moisture generated tend to lower the recoveries by causing the GV to irre-

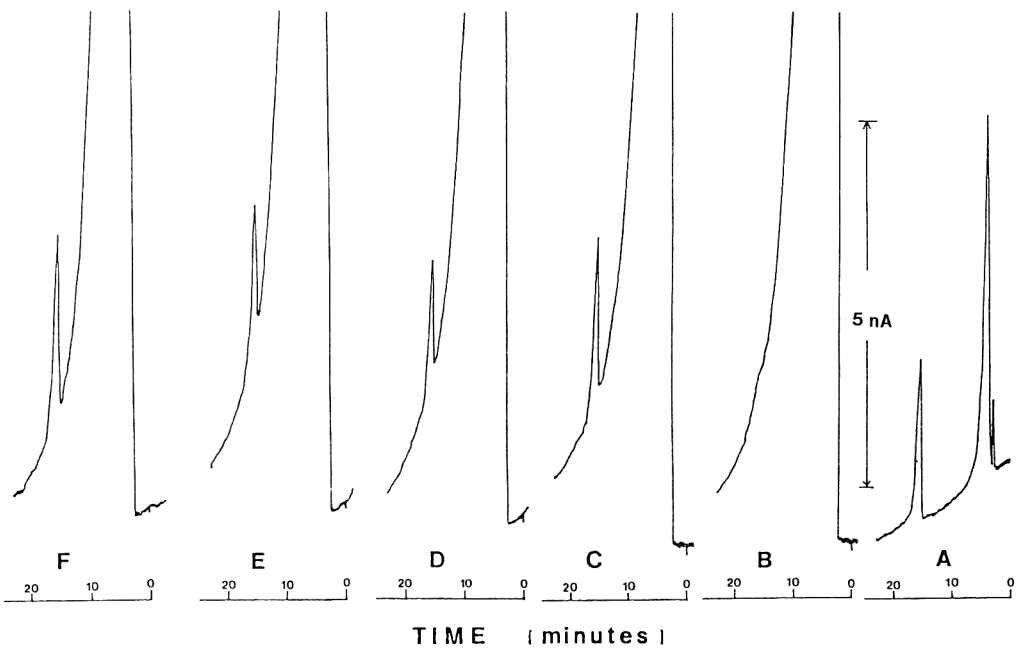

Figure 1. Typical chromatograms from LC/ED determination of GV in fortified feeds: (A) gentian violet standard, 0.1 μg/mL, 20 μL injection (2 ng GV); (B) control chicken feed (5% tallow), 0.04 g feed/mL, 20 μL injection (0.8 mg feed); (C) chicken feed, A-21 (8 ppm), 0.01 g feed/mL, 20 μL injection (0.2 mg feed); (D) chicken feed, A-23 (4 ppm), 0.02 g feed/mL, 20 μL injection (0.4 mg feed); (E) turkey feed (1% tallow), B-22 (4 ppm), 0.02 g feed/mL, 20 μL injection (0.4 mg feed); (F) turkey feed, B-23 (8 ppm), 0.01 g feed/mL, 20 μL injection (0.2 mg feed).

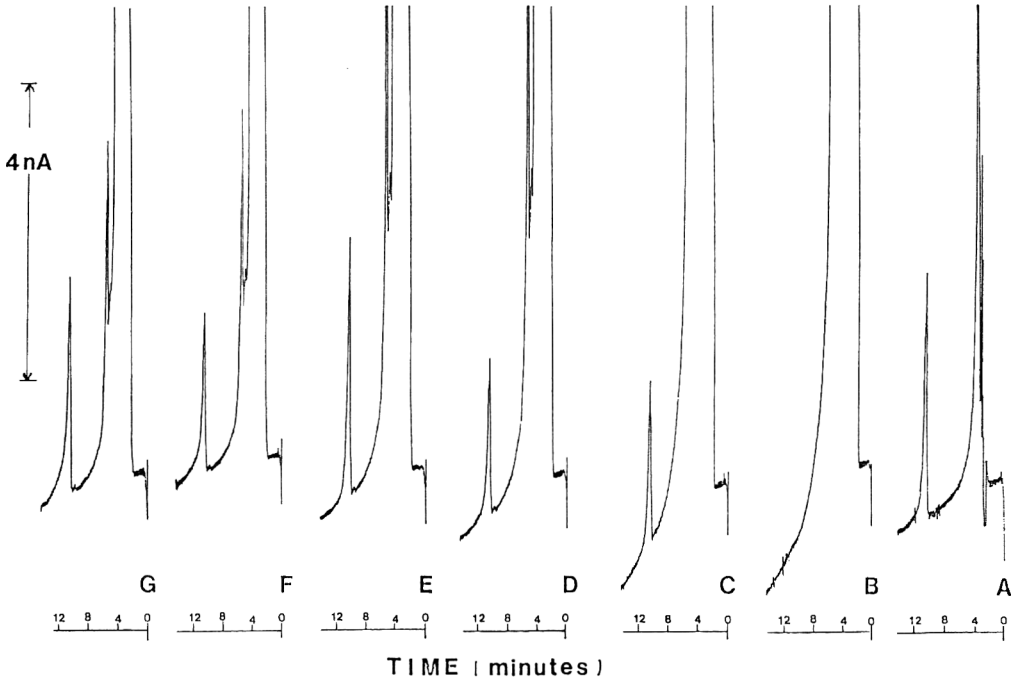

Figure 2. Typical chromatograms from LC/ED determination of GV in fortified chick basal diet (CBD): (A) gentian violet standard, 0.1 μg/mL, 20 μL injection (2 ng GV); (B) control CBD feed, 0.04 g feed/mL, 20 μL injection (0.8 mg feed); (C) spiked CBD, 2 ppm, 0.04 g feed/mL, 20 μL injection (0.8 mg feed); (D) spiked CBD, 4 ppm, 0.02 g feed/mL, 20 μL injection (0.4 mg feed); (E) spiked CBD, 6 ppm, 0.02 g feed/mL, 20 μL injection (0.4 mg feed); (F) spiked CBD, 8 ppm, 0.01 g feed/mL, 20 μL injection (0.2 mg feed); (G) spiked CBD, 10 ppm, 0.01 g feed/mL, 20 μL injection (0.2 mg feed).

versibly stain the matrix. Table 3 shows the effect of grinding on the overall recovery of GV from fortified feeds. A 9% loss in recovery was noted after grinding, with a corresponding 3% increase in CV. Therefore, the choice is between higher recoveries of the assay without grinding and improved CVs with grinding. Figure 1 shows some typical LC/ED chromatograms obtained from analysis of fortified feed.

Our recent acquisition of a Shimadzu SPD-6AV spectrophotometric detector for LC led us to investigate the applicability of this method to visible detection. Earlier work with

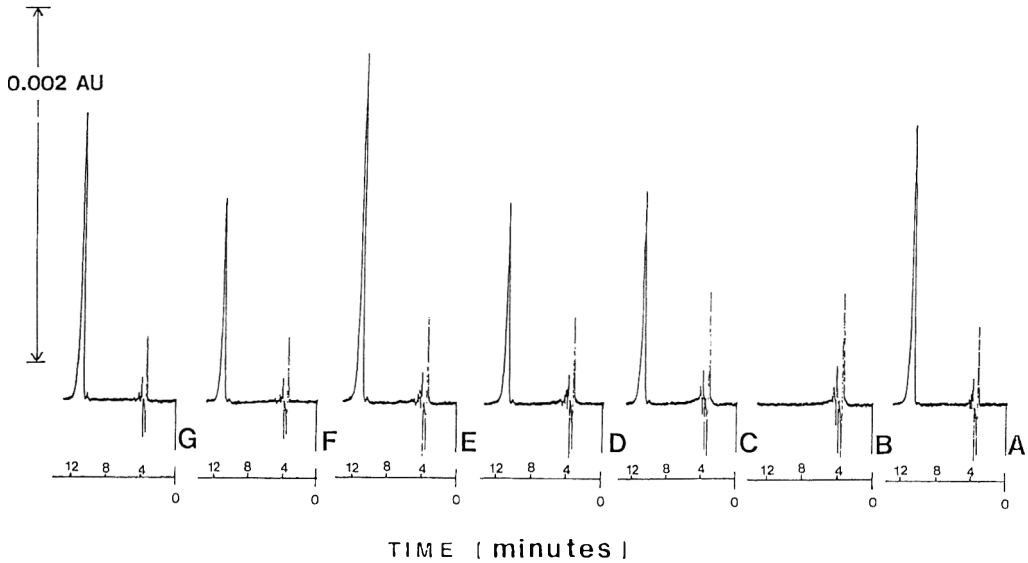

Figure 3. Typical chromatograms from LC/VIS determination of GV in fortified chick basal diet (CBD): (A) gentian violet standard, 0.1 μg/mL, 20 μL injection (2 ng GV); (B) control CBD, 0.04 g feed/mL, 20 μL injection (0.8 mg feed); (C) spiked CBD, 2 ppm, 0.04 g feed/mL, 20 μL injection (0.8 mg feed); (D) spiked CBD, 4 ppm, 0.02 g feed/mL, 20 μL injection (0.4 mg feed); (E) spiked CBD, 6 ppm, 0.02 g feed/mL, 20 μL injection (0.4 mg feed); (F) spiked CBD, 8 ppm, 0.01 g feed/mL, 20 μL injection (0.2 mg feed); (G) spiked CBD, 10 ppm, 0.01 g feed/mL, 20 μL injection (0.2 mg feed).

Table 4. Recovery of GV from fortified chick basal diet[a]

	Rec., %[b]		CV, %	
Fortification level, ppm	LC/ED	LC/VIS	LC/ED	LC/VIS
0	—	—	—	—
2	86	94	7.1	6.0
4	81	90	8.4	8.0
6	86	95	11.2	6.1
8	90	96	11.1	10.7
10	89	95	6.6	4.7
Overall mean rec., %	86	94		
Overall CV, %	3.8	2.7		

[a] Fortified feeds received from CVM, Beltsville, MD.
[b] Average of 5 determinations.

photodiode array and variable wavelength detectors proved them to be unsatisfactory for detection of GV at very low concentrations (<0.1 μg/mL). UV/VIS detectors with a tungsten lamp light source appear to be very well suited for use in this analysis. For a UV/VIS spectrophotometer to be acceptable, a 20 μL injection of a GV solution containing 0.1 μg/mL (2 ng GV) should give a minimum peak response of 1.5 mAU. Figures 2 and 3 are typical LC/ED and LC/VIS chromatograms, respectively. Table 4 shows recovery data obtained with each detector in analysis of fortified chick basal diet. Although LC/ED is an excellent system for the simultaneous determination of residue levels of GV, its demethylated metabolites, and leucogentian violet in tissue, the LC/VIS detector is quite satisfactory for feed analyses and is preferred. Because no extensive cleanup procedures are involved, the LC/VIS detector is less prone to fouling, and therefore requires less maintenance.

Electrochemical (+1.000 V) or visible detection (588 nm) in combination with LC results in a simple and solvent-efficient analytical tool. The method eliminates the use of hazardous solvents, such as benzene and toluene, because the time-consuming Sephadex column chromatography step is not used. Compared with previously published methods, the method as developed and presented provides a more rapid chromatographic determination of gentian violet in poultry feeds at therapeutic levels of 4–8 ppm.

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FOOD ADDITIVES

Solid-Phase Extraction Method for Volatile *N*-Nitrosamines in Hams Processed with Elastic Rubber Netting

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A method was developed for the determination of volatile *N*-nitrosamines in hams processed in elastic rubber nettings. The method was based on a modification of a solid-phase extraction (SPE) procedure used in the past to determine selected nitrosamines in different types of cured meat products. The nitrosamines detected in ham most likely originate from the amine precursors in rubber and from the nitrite commonly used in the meat curing process. The method was compared with 2 established procedures for *N*-nitrosodibutylamine (NDBA) analysis in cured meat products: the mineral oil distillation procedure (MOD) and the low temperature vacuum distillation procedure (LTVD). All 3 methods used the same gas chromatographic/chemiluminescent detection conditions and system. No significant difference was found between the MOD and LTVD methods. These methods were found to yield significantly higher NDBA levels than the SPE procedure. When 2,6-dimethylmorpholine was added to the sample before analysis in the MOD and LTVD procedures, artifactual nitrosamines were formed. No artifactual formation was noted in the SPE method. We propose that the new SPE method replace the current methods being used for analysis of netted, cured meat products.

Fajen et al. (1) first reported volatile *N*-nitrosamines in the air of rubber and tire manufacturing plants in 1979. Since then, several reports have been published about detection of nitrosamines in the precursors used in the production of natural and synthetic rubber (2, 3) and in the finished products themselves. The source of these nitrosamines was attributed to rubber vulcanization accelerators, which contain a dialkylamine or acyclic amino group. The nitrosamines found range from simple dialkyls such as *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), and *N*-nitrosodibutylamine (NDBA) to alicyclics such as *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR), and *N*-nitrosomorpholine (NMOR). For instance, Lakritz and Kimoto (4) reported nitrosamines in rubber-stoppered blood collection tubes, and Fiddler et al. (5) found them in disposable rubber gloves. Ireland et al. (6) found nitrosamines in a wide variety of finished rubber products including gloves and condoms. The most widely publicized reports concerned their detection in infant pacifiers and baby bottle nipples (7–9). Regulatory action initially limited the total nitrosamine content in the rubber nipples to 60 ppb (10). This was eventually lowered to 10 ppb, and the rubber industry complied with this limit (11). A similar reduction in nitrosamine content of nipples and pacifiers was observed by Sen et al. in Canadian investigations (12, 13).

The finding of nitrosamines in rubber products raised concern about the possible hazards of rubber-containing products in contact with food and the possible migration of preformed nitrosamines into the food. Sen et al. (14) reported finding NDEA and NDBA in cured meats held in elastic rubber netting during smokehouse processing. They found trace quantities of these nitrosamines in the unused netting and high levels (up to 504 ppb NDBA) in the used netting. The corresponding meat samples also contained NDBA (up to 29 ppb). Recently, the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture, while examining a new process for preparing hams, found significant levels of NDBA in the product. This was attributed to rubber in the elastic netting.

A comprehensive FSIS monitoring program of hams and other products processed in these elastic rubber nettings is expected to determine the extent of the occurrence of nitrosamines, before regulatory action. However, the available methodology creates problems with conducting an extensive survey. First, only limited numbers of samples can be analyzed by methods currently in use by FSIS. Second, the reliability of this methodology needs to be demonstrated for volatile nitrosamines, particularly NDBA. Third, the simultaneous presence of both nitrite in the cured meat product and amine from the rubber, which may have migrated into the meat product, may artifactually produce NDBA as a result of analysis (14). Therefore, an alternative method based on a nondistillation technique had to be developed to minimize the potential for artifact formation. This method was then compared with those currently in use.

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Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

METHOD

Caution: *N*-Nitrosamines are potential carcinogens. Exercise care in handling these compounds.

Reagents

(a) *Celite 545.*—Not acid-washed (Fisher Scientific Co.). Test reagent blank before starting sample analysis, particularly if new lot of Celite is used. If interfering chromatographic peaks are noted, prewash twice with dichloromethane (DCM), filter, then dry 4 h in 120°C vacuum oven before use.

(b) *Sodium sulfate.*—Anhydrous, granular (Mallinckrodt No. 8024).

(c) *Silica gel.*—70–230 mesh (EM 7734). Prewash twice with DCM, filter, and dry 4 h in 60°C vacuum oven before use.

(d) *Propyl gallate.*—Aldrich Chemical Co.

(e) *Morpholine.*—Aldrich Chemical Co., doubly distilled before use to remove traces of NMOR.

(f) *2,6-Dimethylmorpholine.*—Aldrich Chemical Co., checked for nitrosamine contamination before use.

(g) *Dichloromethane (DCM), pentane, ethyl ether.*—LC grade (Burdick and Jackson).

(h) *N-Nitrosodipropylamine (NDPA) internal standard solution.*—0.10 μg/mL in DCM.

(i) *Gas chromatography working standard solution.*—NDMA, *N*-nitrosomethylethylamine (NMEA), NDEA, NDPA, *N*-nitrosoazetidine (NAZET), NDBA, NPIP, NPYR, NMOR, and *N*-nitrosohexamethyleneimine (NHMI), each 0.10 μg/mL in DCM. These nitrosamines were either purchased or synthesized from their corresponding amines and sodium nitrite according to general procedure published previously (15). *N*-Nitroso-2,6-dimethylmorpholine was also synthesized as above.

(j) *Ham samples.*—Random samples were obtained from local suppliers or FSIS and analyzed without further heating. Two samples were obtained from each ham: outer 1/4 in. and second 1/4 in. of product. Grind samples through 1/16 in. plate before analysis and store in −20°C freezer until analyzed.

Apparatus

(a) *Mortar and pestle.*—Glass, 473 mL (16 oz., A.H. Thomas).

(b) *Chromatographic columns.*—(*1*) Glass, 350 × 32 mm id with 60 × 6 mm id drip tip, no stopcock, prepared by glassblower. (*2*) Glass, 300 × 19 mm with 250 mL reservoir (Lurex Scientific).

(c) *Tamping rod.*—Glass, 450 mm long with 12 mm diameter disk on end, prepared by glassblower.

(d) *Evaporative concentrator.*—Kuderna-Danish (K-D), 250 mL; concentrator tube, 4 and 10 mL, Snyder (3-section) and micro-Snyder distilling columns (Kontes Glass Co.).

(e) *Gas chromatograph-Thermal Energy Analyzer (GC-TEA).*—Shimadzu gas chromatograph Model GC-14A equipped with AOC-14 auto-injector or equivalent, interfaced to Thermal Energy Analyzer Model 502A (Thermedics, Inc.). Operating conditions: 2.7 m × 2.6 mm glass column packed with 15% Carbowax 20M-TPA on 60–80 mesh Gas Chrom P; He carrier gas 35 mL/min; injector 180°C; TEA furnace 475°C; TEA vacuum 0.4 mm; liquid nitrogen cold trap; column programmed from 120 to 200°C at 4°C/min.

Determination

(a) *Solid-phase extraction (SPE).*—Weigh 10.0 ±0.1 g sample into mortar, and add 250 mg propyl gallate directly to sample. Spike sample with 1.0 mL internal standard solution (equivalent to 10 ppb), using transfer pipet. Add 25 g anhydrous sodium sulfate and mix with pestle ca 15 s; then add 20 g Celite and again mix with pestle 15–20 s until Celite is thoroughly mixed with sodium sulfate and sample. Grind entire mixture with moderate pressure for additional 1 min. Using powder funnel, quantitatively transfer mixture into glass column (350 × 32 mm) containing glass wool plug at bottom. Tamp with tamping rod to achieve height of ca 75 mm. Add 20 g anhydrous sodium sulfate to top of column. Rinse mortar, pestle, and tamping rod with 20 mL DCM and add rinse to top of column. Immediately add additional 130 mL DCM to column (column will darken when solvent elutes through it). Collect eluate in 250 mL K-D flask equipped with 10 mL concentrator tube. When column stops dripping, remove K-D flask (discard contents of glass column), add boiling chip, attach Snyder column, and concentrate eluate on steam bath until DCM stops distilling. There will be ca 3–7 mL of concentrate remaining in concentrator tube. Add 4.0 g silica gel to glass column (300 × 19 mm with 250 mL reservoir) containing glass wool plug and 25 mL pentane, and top it with 5.0 g anhydrous sodium sulfate. Using disposable glass pipet, quantitatively transfer concentrate to silica gel column; then rinse concentrator tube with two 4 mL portions of pentane and add to column. Collect eluate in 250 mL Erlenmeyer flask (flow rate ca 2–3 drops). When liquid level in column reaches top of sodium sulfate, add 150 mL wash mixture (25% DCM in pentane). When liquid level in column again reaches top of sodium sulfate, change collection vessel to 250 mL K-D flask equipped with 4 mL concentrator tube (discard contents of Erlenmeyer flask). Add 150 mL elution solvent (30% ether in DCM). When column stops dripping, remove K-D flask, add boiling chip, attach Snyder column, and concentrate on steam bath to 4 mL. Remove Snyder column and K-D flask, add new boiling chip, attach micro Snyder column, and concentrate to 1.0 mL in 70°C water bath. Do not concentrate sample with stream of nitrogen. (**Note**: Room temperature should be less than 24°C during SPE procedure.)

(b) *Low temperature vacuum distillation (LTVD).*—Samples were analyzed by technique developed by Sen et al. (16) and described in detail in *USDA, FSIS Chemistry Laboratory Guidebook* (17). Briefly, 25 g sample, without any nitrosation inhibitors, was distilled under vacuum (20 torr) from base in 2 L pear-shaped flask immersed in 45–46°C water bath. Aqueous distillate was acidified and extracted with DCM. DCM was washed with acid and base, dried with anhydrous sodium sulfate, and concentrated.

(c) *Mineral oil distillation (MOD).*—Samples were analyzed by method originally developed by Fine et al. (18) as specified in *USDA, FSIS Chemistry Laboratory Guidebook*

Table 1. Recovery of volatile *N*-nitrosamines in ham at the 10 ppb fortification level

N-Nitroso compound	Recovery, %			
	Range	Mean (n = 12)	SD	CV
Dimethylamine	67.8–96.0	81.7	8.7	10.7
Methylethylamine	67.0–91.0	77.9	6.4	8.3
Diethylamine	68.9–90.5	77.8	6.9	8.9
Dipropylamine	71.4–108.2	89.6	10.5	11.7
Azetidine	77.5–105.4	91.7	8.6	9.4
Dibutylamine	72.0–102.4	87.7	11.2	12.8
Piperidine	85.8–105.6	96.0	6.7	7.0
Pyrrolidine	83.3–109.1	97.5	9.1	9.3
Morpholine	81.2–102.4	94.8	7.4	7.8
Hexamethyleneimine	85.6–109.5	99.5	7.6	7.7

(19). Briefly, 25 g sample, without any nitrosation inhibitors, was distilled under vacuum (<2 torr) from base and mineral oil to temperature of 120°C. Aqueous distillate was extracted with DCM, dried with anhydrous sodium sulfate, and concentrated.

(d) *Nitrosamine determination.*—Quantitate volatile nitrosamines as described previously (20), using 5.0 µL injection. Minimum detectable level (signal:noise >2) of NDMA, NMEA, and NDEA, 0.2 ppb; NAZET, NPIP, NPYR, NMOR, and NHMI, 0.5 ppb; and NDBA, 1.0 ppb.

(e) *Sodium nitrite analysis.*—Residual sodium nitrite was determined in 10.0 g sample by Griess-Saltzman procedure as modified by Fiddler (21).

(f) *Statistical analysis.*—Data were analyzed by General Linear Model and Means procedures (ANOVA and Student's paired t-test) of Statistical Analysis System PC software distributed by SAS Institute, Inc. (22). These results were then interpreted according to methods of Snedecor and Cochran (23) and Youden and Steiner (24).

Results and Discussion

There is an ongoing need to improve and expand the capabilities of the methodology used in the analysis of cured meat products for volatile nitrosamines, with assurance that nitrosamines will not artifactually form during analysis. We have previously shown that our solid-phase extraction (SPE) procedure is versatile. It enabled us, with solid support and solvent modifications, to determine NPYR in pumped and dry-cured bacon (20, 25) and nitrosoamino acids in a variety of cured meat products (26). The analysis of frankfurters containing fish protein in the form of Alaska pollock mince and surimi posed a special problem with regard to artifactual NDMA formation because of the presence of both nitrite in the meat and dimethylamine in the fish. This problem was resolved by using 2 chromatographic columns. In the first, the amine and nitrosamine were separated from the nitrite-containing sample, and in the second, the nitrosamine was isolated from the retained amine (27). Although this SPE method has been used for the isolation and quantitation of selected nitrosamines in specific sample types, its potential applicability has not been fully investigated. For example, the SPE method with acid-Celite in the bottom column of a 2-column system could only be used to isolate NDMA, NAZET, NPYR, and NMOR, because of the acidified Celite's retention characteristics. To isolate any other nitrosamines, a third column containing silica gel or alumina was required (20, 27). The use of a second column containing silica gel was based on a modification of the method originally developed by White et al. (28). Because NDBA was the nitrosamine of primary interest in the elastic-netted cured meat products, a modification in the solid support was required. First, changing the acid-to-Celite ratio was tried, but NDBA was not retained. Next, substituting silica gel for acid-Celite in the lower column was attempted. The amount of silica gel in the lower column and the solvent system used to elute the NDBA contained in the lipids were both varied, but there was still too much lipid material in the extract for quantitation to be practical. Therefore, this approach was abandoned. The use of an SPE column containing the meat sample, anhydrous sodium sulfate, and Celite with direct DCM extraction followed by a separate silica gel column was found to give the best results.

A ruggedness test of the SPE procedure was performed on ham containing naturally incurred 15.6 ppb NDBA. Deviations in the normal grinding, packing, and solvent elution steps in the first column and packing and elution steps in the second column indicated that the results were not significantly different except for the effect of room temperature. When the room temperature exceeded 24°C, the use of the pentane-containing solvent system with the silica gel column caused separating and channeling. This resulted in lower recoveries of both NDBA and the internal standard, NDPA. In addition, during the development of this method, 50 ppm morpholine, a rapidly nitrosated amine, was added to the sample before analysis to assess artifact formation; no NMOR was detected.

The recoveries of 10 volatile *N*-nitrosamines added to nitrosamine-free ham at the 10 ppb level are shown in Table 1. Recovery of NDBA, the nitrosamine commonly found in netted hams, was 88%. The mean recovery of all other nitrosamines was >78%. Statistical analysis of the data by Student's paired t-test showed no significant difference in recovery between NDPA and NDBA ($P < 0.05$, $n = 12$). For this reason, and be-

Table 2. Determination of *N*-nitrosodibutylamine in netted ham by 3 methods

		SPE[a]		LTVD[a]		MOD[a]	
Sample	$NaNO_2$, ppm	NDPA, %	NDBA, ppb[b]	NDPA, %	NDBA, ppb[b]	NDPA, %	NDBA, ppb[b]
A	ND[c]	83.7	15.8	87.8	19.6	106.0	18.7
B	1.0	87.9	22.2	89.8	28.8	101.3	33.0
C	1.5	104.6	41.8	84.6	53.6	83.1	55.3
D	1.6	86.3	14.4	95.1	26.6	112.9	24.5
E	1.9	95.9	19.9	88.5	23.6	83.4	23.4
F	2.1	80.5	49.9	97.5	50.9	97.3	37.3
G	2.5	85.3	26.3	85.6	30.2	95.6	32.1
H	3.5	94.5	17.8	88.1	15.8	97.2	17.9
I	5.1	90.0	10.8	81.4	10.7	98.8	10.6
J	7.6	93.9	22.5	87.5	22.9	102.6	26.0
K	10.1	84.3	50.1	83.4	43.5	98.7	54.6
L	12.1	95.7	22.4	85.0	28.6	89.0	30.7
M	12.7	83.1	18.2	92.0	15.3	104.6	18.2
N	13.4	89.6	11.1	91.7	9.8	97.4	10.4
O	16.0	83.9	14.0	101.3	16.6	92.5	15.0

[a] Results are averages of duplicate determinations.
[b] Data corrected for recovery of the NDPA internal standard.
[c] ND, none detected, <1 ppm.

cause NDPA has not been reported in any food or rubber products and is used as the internal standard in the MOD and LTVD methods, it was chosen as the internal standard for our SPE procedure.

After the reliability of the SPE procedure was determined, NDBA was determined in commercial ham samples in duplicate by each of 3 methods: SPE, MOD, and LTVD. Results, averaged over 2 determinations, are shown in Table 2. Residual sodium nitrite was also determined in all 15 hams. No statistical correlation ($P < 0.05$) was found between residual nitrite and NDBA values in any of the methods. Individual NDBA values ranged from 10.3 to 51.2 ppb for SPE, 9.6 to 54.8 ppb for LTVD, and 10.3 to 58.3 ppb for MOD. Mean recoveries for the internal standard were 89.3, 89.3, and 97.3% for the SPE, LTVD, and MOD methods, respectively. Data were analyzed by ANOVA, and the means of the methods were further examined by Duncan's multiple range test at the $P < 0.05$ level. The repeatibilities were as follows: 1.3 ppb, CV 6.2% (0.7 ppb, CV 2.8%, corr.) for the SPE procedure; 2.65 ppb, CV 11.2% (1.5 ppb, CV 5.8%, corr.) for the LTVD procedure; and 1.6 ppb, CV 6.0% (2.26 ppb, CV 8.3%, corr.) for the MOD procedure.

As shown in Table 3, with the uncorrected data, the methods were significantly different from each other. With the data corrected for the recovery of the internal standard, no significant difference between the MOD and LTVD was detected; however, the SPE differed significantly from both. The MOD and LTVD values in both the uncorrected and corrected data were higher than the SPE data. This suggests artifactual formation of NDBA during the MOD and LTVD sample analysis. The procedures currently being used by FSIS to determine NDBA in ham samples (MOD and LTVD) do not use any nitrosation inhibitors during analysis. The SPE procedure uses propyl gallate to inhibit artifact formation. The MOD and LTVD methods rely on alkalinization to prevent artifact formation during distillation, but Challis and Kyrtopoulos have shown that nitrosation can occur even under alkaline conditions (29).

To determine whether nitrosamines could form artifactually in any of these procedures, 50 ppm 2,6-dimethylmorpholine, a rapidly nitrosated secondary amine, was added to several ham samples before analysis. *N*-Nitroso-2,6-dimethylmorpholine was detected in 11 of 11 samples analyzed by the MOD method (7.0–492.0 ppb; mean, 73.1 ppb) and in 4 of 6 samples analyzed by the LTVD method (8.5–36.8 ppb; mean, 23.8 ppb). None was detected in 10 of 10 samples analyzed by the SPE procedure. Artifactual nitrosamine formation during MOD analysis was previously demonstrated when additional nitrite or amine was added to the cured meat samples before analysis (20, 24, 30). There is an indication that nitrosating species can be generated in cured meat products even if the measured residual nitrite is low or not detected. For example, Hotchkiss et al. (31) demonstrated that lipid–nitrite reaction products have nitrosative ability, and others have successfully formed nitrosamines by transnitrosation of nitrosothiols (29, 32). Therefore, artifact formation during MOD and LTVD was not completely unexpected.

Table 3. Comparison of the 3 methods for analysis of NDBA in hams

	n	SPE	LTVD	MOD
Mean	30	21.23[a]	23.54[b]	26.12
Mean (Corr.)	30	23.80[a]	26.43[b]	27.19[b]

[a] Not significant y different ($P < 0.05$) from each other.
[b] Not significantly different ($P < 0.05$) from each other.

In conclusion, the newly developed SPE procedure is distillation-free, offers an opportunity to perform more analyses than the current methods, and gives good recoveries for a wide variety of volatile nitrosamines. It is not susceptible to artifactual nitrosamine formation, as might occur when the sample contains either high levels of residual nitrite or a nitrosamine precursor. Therefore, we propose that this SPE procedure represents a reliable alternative to the MOD and LTVD methods for determining nitrosamines in cured meat products processed in elastic rubber nettings.

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FOOD COMPOSITION

Determination of Mono- and Disaccharides in Foods by Interlaboratory Study: Quantitation of Bias Components for Liquid Chromatography

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In 1988, a group of French food manufacturers decided to validate the methods used to determine mono- and disaccharides in foods (fructose, glucose, sucrose, maltose, and lactose) based on liquid chromatography with refractive index detection. Twenty laboratories analyzed 12 products. The reproducibilities obtained exceeded Horwitz's criteria but were still acceptable because laboratories used different equipment and the sample matrixes were complex. Six different sources of variation were characterized because of a more complex experimental design. The method for calculating concentration, in particular, had considerable impact on the final precision; the choice between peak area and peak height must be strictly considered.

More than 10 official methods are available for the analysis of mono- and disaccharides in foods (1). However, these methods, listed in Table 1, do not give very consistent nutrient data and make it difficult to evaluate food contents precisely. A recent publication on food nutrition labeling discussed the precision of analytical methods used to determine the major nutrients. The present procedures for the analysis of mono- and disaccharides in foods were shown to be not really adapted for this determination (2). For example, the precision of liquid chromatographic (LC) determination of sugars in cereal products for concentrations below 30 g/100 g was unacceptable. It was suggested that the heterogeneity of solid test samples induced an important variability.

In 1988, a group of French food manufacturers decided to evaluate the present carbohydrate consumption of French consumers. As a preliminary step, it appeared necessary to validate the methods used to determine mono- and disaccharides in foods. The next step consisted of collecting composition data on various foods and performing a consumption survey. Thus, 5 sugars were selected (fructose, glucose, sucrose, maltose, and lactose) according to their industrial and nutritional importance, and several interlaboratory studies were organized. It was decided to use an analytical method based on LC with refractive index detection; the equipment is accessible and already used by most laboratories, and the detection limit is well-adapted to the expected concentration levels.

Four food groups have been studied, representing more than 60% of the total sugar production used by the food industry in France: fresh dairy products; cereal products; soft drinks; and confectionery and chocolate products. Because the results obtained for fresh dairy products have already been published elsewhere (3), this paper will present only the results for the 3 other food groups.

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Experimental

The principles of the method itself are very similar to those of the AOAC method (4), especially for chocolate products. The main differences concern sample preparation; modifications were introduced to shorten this time-consuming step. Sample preparation was standardized because we assumed that this step represents the major source of variation.

Apparatus

For the stationary phase, amino-bonded silica gel was generally preferred to cation exchange resin. Amino-bonded silica gel columns give an optimal separation of disaccharides (sucrose, maltose, and lactose) and allow the use of Carrez reagent for defatting; this method is simpler than centrifugation and decantation and has been adopted by the International Office of Cocoa, Chocolate, Confectionery as an official method (5). However, separation efficiency is highly variable from one column to another, and column lifetime is shortened by the development of Shiff bases through the interactions of the sugars and the stationary phase. Thus, the column separation performance might be regularly controlled by using the AOAC resolution factor between fructose and glucose peaks.

Reagents

The eluant composition was adjusted according to the column age and/or brand; the acetonitrile–water ratio varied from 65 + 35 (v/v) for a new column to 85 + 15 for an old one. The value of 75 + 25 was recommended, but each participant made his or her own adjustment.

Table 1. Principle references of AOAC sugar methods of analysis, 15th edition, 1990

Sugar	Food	Method	Reference
Sucrose	Cordials, liqueurs	Polarization, reduction	**940.11**
Reducing sugars	Beer	Layne-Eynon, Munson-Walker	**920.51**
Reducing sugars	Wine	Munson-Walker	**920.64**
Sucrose	Wine	Polarization, reduction	**920.65**
Commercial glucose	Wine	Polarization	**920.66**
Glucose and fructose	Wine	Enzymatic	**985.09**
Sucrose	Nonalcoholic beverage	Polarization, reduction	**950.29**
Reducing sugars	Nonalcoholic beverage	Reduction	**950.30**
Fructose, α β glucose, sorbitol, sucrose	Fruits, juice	Gas chromatography	**971.18**
Sucrose	Fruits	Polarization, reduction	**925.35**
Sugars	Roasted coffee	Reduction, titration	**925.15**
Lactose	Milk chocolate	Titration	**933.04**
Fructose, glucose, maltose, sucrose, lactose	Milk chocolate	Liquid chromatography	**980.13**
Sucrose	Cacao products	Polarization	**920.82**
Glucose	Cacao products	Zerban-Sattler	**938.18**
		Sichert-Bleyer	**936.06**
Sugars	Flour	Titration	**939.03**
Glucose, fructose	Presweetened cereals	Liquid chromatography	**982.14**
Sucrose and maltose reducing sugars	Bread	Munson-Walker	**975.14**
Lactose	Bread	Titration	**952.05**
Lactose	Milk	Polarization	**896.01**
		Gravimetry	**930.28**
		Near infrared	**972.16**
		Enzymatic	**984.15**
Lactose	Meat	Benedict solution	**927.07**
Reducing sugars	Honey	Layne-Eynon, Munson-Walker	**920.183**
Fructose, glucose, sucrose		Charcoal column chromatography	**954.11**
Fructose, glucose, sucrose		Liquid chromatography	**977.20**

Carrez Solutions I and II were used for sample preparation. Solution I consisted of a saturated solution of potassium hexacyanoferrate (II) in distilled water; Solution II was prepared from a saturated solution of zinc acetate in distilled water.

Sample Preparation

(a) *Soft drinks.*—For ready-to-drink beverages, pulpy samples were homogenized and fizzy samples degassed 20 min/L in an ultrasonic bath. About 10 g was accurately weighed in a 100 mL flask. Pulpy samples were filtered on rapid paper and through a 0.47 μm membrane.

For syrups or concentrated drinks, uronic compounds, such as pectins and gums, were removed by alcoholic extraction; ca 10 g was accurately weighed in a 100 mL flask, diluted to 100 mL with ethanol, mixed thoroughly, and cooled 5 min to 5°C. The solution was filtered on rapid paper, diluted with distilled water to a controlled dilution ratio to obtain a concentration within the range for standard solutions, and finally filtered through a 0.47 μm membrane.

(b) *Cereals and chocolate and confectionery.*—The whole sample was homogenized (size ranged from 100 g for candy to 1 kg for heterogeneous cake). Sticky samples, such as jam or caramel, had to be frozen before grinding. Samples ranging from 5 g for a biscuit to 2 g for confectionery were accurately weighed in a 50 mL flask. About 30 mL hot water (ca 40°C) was added, and the solution was heated 15 min in a water bath at 60°C. The flask was continuously swirled while adding 2 mL of each Carrez solution. The solution was then cooled to room temperature, diluted to volume with water, and filtered through a 0.47 μm membrane.

Chromatographic Conditions

Flow rate and column length depended on the apparatus model. For a porosity of 5 μm and a flow rate of 1 mL/min, separation time was ca 25 min. Participants were requested to calculate concentrations by using both peak area and peak height. Laboratories could use an internal standard and compute their results from either an internal or external standard.

Design of Interlaboratory Studies

Four interlaboratory studies were organized from 1988 to 1990, in which 20 laboratories participated. Results of the first

Table 2. Products and sugars analyzed

1. Soft drinks	2. Cereal products	3. Chocolate, confectionery
D1: Lemon soft drink	C1: Crispbread	S1: Nougat-filled chocolate
D2: Cola drink	C2: Sweet biscuit	S2: Sweet
D3: Orange juice, 20%	C3: Chocolate biscuit	S3: Rocher cream
D4: Pure orange juice		S4: Chocolate bars
D5: Orange syrup		

Sugars		
fr: Fructose	gl: Glucose	su: Sucrose
ma: Maltose	la: Lactose	

Table 3. Apparatus of participating laboratories

Laboratory	Pump	Automatic sampler	Detector	Integrator	Acquisition program
		Study No. 1. Soft drinks			
1	Varian 5000	none	Varian RI-3	Varian Vista 402	none
2	Gilson 302	none	Varian RI-4	SP 4290	none
3	Bischoff 2220	none	Bischoff RI 8100	Shimadzu CR3A	yes
4	Waters 510	Waters WIPS	Waters 410	Waters 740	none
5a	Varian 2010	Varian 8085	Varian RI-3	Varian Vista 402	none
5b	Varian 2010	Varian 8085	Varian RI-3	Varian Vista 402	none
6	SP 8800	yes	Knauer RI	SP 4290	yes
7a	Waters 510	SP 8875	Waters R 401	Merck D 2000	none
7b	Waters 510	SP 8875	Waters R 401	Merck D 2000	none
8	SP	SEDERE	SP	Shimadzu CR3A	none
9	Waters 501	none	SOPARES 7510	Shimadzu CR3A	none
		Study No. 2. Cereal products			
3	Bischoff 2220	none	Bischoff RI 8100	Shimadzu CR3A	STACQ
6	SP 8810	none	Knauer RI	SP 4290	WINNER
9	Waters 501	none	SOPARES 7510	Shimadzu CR3A	none
10	Waters M-600	none	Shimadzu RID-6A	SEFRAM Servotrace	none
11	SP 8800	SP 8775	SP 8490	SP 4290	WINNER
12	Waters M 510	none	Waters RI 410	SP 4290	LNT
14	Shimadzu LC6A	none	Shimadzu RID-6A	Shimadzu CR3A	none
		Study No. 3. Chocolate-confectionery products			
3	Bischoff 2220	none	Bischoff RI 8100	Shimadzu CR3A	STACQ
4	Waters 510	WIPS	Waters RI 410	Data Module 740	none
6	SP 8800	Gilson 231	Knauer RI	SP 4290	WINNER
9	Waters 6000A	none	SOPARES 7510	Shimadzu CR3A	none
11	SP 8800	SP 8775	SP 8490	SP 4290	WINNER
12	Waters M 510	none	Waters RI 410	SP 4290	LABNET
15	Varian 2510	none	Waters RI 401	Varian 4290	none
16	SP 8700	none	Spectra 6040 XR	Spectra 4270	WINNER
17	Perk Elmer S2	none	RI ERC 7512	HP 3390 A	none
18	SP 8770	none	Shodex RI SE61	SP 4290	none
19	Waters 510	none	Waters RI	Waters 410	none
20	Waters 510	none	Waters RI 410	Waters 740	none

Table 4. Chromatographic conditions

Laboratory	Column	Loop, μL	CH_3CN–H_2O	Flow, mL/min	Length, m	Internal standard
		Study No. 1. Soft drinks				
1	Beckman NH_2 5 μm	10	75 + 25	1	13	none
2	NH_2 8 μm	20	70 + 30	1.5	10	rhamnose
3	Bioblock NH_2 5 μm	20	75 + 25	1	25	xylose
4	Interchim L7 NH_2-25 F	20	75 + 25	1.5	12	none
5a	CHO 620	10	0 + 100	0.3	30	mannitol
5b	NH_2 10 μm	10	75 + 25	1.3	15	rhamnose
6	Lichrosorb NH_2 5 μm	10	80 + 20	1	16	none
7a	Sugar Pak I	10	0 + 100 + Ca	0.5	16	none
7b	Merck D 2000 SFCC NH_2 5 μm	10	75 + 25	1	20	none
8	Chrompack NH_2 10 μm	10	75 + 25	1	27	none
9	Lichrosorb NH_2 5 μm	10	75 + 25	1	16	none
		Study No. 2. Cereal products				
3	Bioblock NH_2 5 μm	20	75 + 25	1	25	none
6	Lichrosorb NH_2 5 μm	10	75 + 25	1	20	none
9	Lichrosorb NH_2 5 μm	20	70 + 30	1	14	none
10	Touzart Matignon NH_2 5 μm	20	75 + 25	1.2	25	none
11	Nucleosil SFCC NH_2 5 μm	20	75 + 25	1	20	none
12	Lichrosorb Merck NH_2 5 μm	10	75 + 25	1.5	10	xylose, 2 g/L
14	Lichrosorb NH_2 5 μm	20	75 + 25	1	17	none
		Study No. 3. Chocolate-confectionery products				
3	Interchim NH_2 7 μm	20	85 + 15	1	30	xylose, 2 g/L
4	Interchim NH_2 7 μm	—	85 + 15	1.5	25	none
6	Lichrosorb Merck NH_2 5 μm	20	75 + 25	0.9	30	xylose
9	Merck NH_2 5 μm	5	70 + 30	0.7	13	none
11	Nucleosil SFCC NH_2 5 μm	20	75 + 25	1	16	none
12	Lichrosorb Merck NH_2 7 μm	20	78 + 22	1.5	13	xylose, 2 g/L
15	Spherisorb NH_2 5 μm	10	75 + 25	1.5	15	xylose, 1 g/L
16	Spherisorb NH_2 5 μm	10	70 + 30	1	30	none
17	Spherisorb NH_2 5 μm	20	82.5 + 17.5	1.5	20	none
18	Lichrosorb Merck NH_2 5 μm	10	75 + 25	1	15	xylose, 1 g/L
19	Lichrosorb Merck NH_2 5 μm	20	75 + 25	1	15	none
20	YCN NH_2	20	75 + 25	2.0	15	none

study for fresh dairy products have already been published (3). The 12 food products analyzed in the 3 studies are listed in Table 2. Food and analyte codes used for other figures and tables are also given in Table 2. Apparatus and operating conditions for each participant are reported in Tables 3 and 4, where laboratories are indicated by number. Because Laboratories 5 and 7 performed analyses twice, with a cation exchange column (codes 5a and 7a) and a silica-bonded column (codes 5b and 7b), they are considered as 4 participants. All other analyses were performed with an amino-bonded column.

For difficult sample preparation, such as cereal and chocolate and confectionery products, 2 replicate preparations (called extractions) were requested. Each extraction was then separately injected twice. For soft drinks, a single extraction was performed but injected 3 times. Thus, the number of replicates was 3 or 4.

Statistical Treatment of Outliers

Precision criteria to validate a method of analysis were computed according to the statistical treatment recommended by international guidelines (6, 7). Within that context, outliers were identified by either the Cochran test or the Grubbs test.

However, the experimental design of the study gave 3 or 4 replicates for each cell; thus, it was necessary to add a preliminary step for the rejection of outlying replicates. The Dixon test was preferred because it is generally considered more powerful with a small number of degrees of freedom. However, it has 2 important drawbacks that may lead to the detection of

Table 5. Raw data (g/100 g) for lemon soft drink (D1)

Laboratory	Area			Height		
	Fructose					
1	0.90	0.92	0.86	0.89	0.92	0.89
2	1.06	1.18	—	1.31	1.42	—
3	0.99	1.06	0.86	1.01	1.09	0.96
4	1.04	1.07	—	1.16	1.17	—
5a	0.80	0.83	0.91	—	—	—
5b	0.66	0.68	0.68	0.73	0.70	0.72
6	0.71	0.65	0.65	0.75	0.71	0.72
7a	0.96	0.95	0.90	0.96	0.97	0.95
7b	0.87	1.16	0.89	0.94	1.01	0.92
8	0.82	0.80	0.82	0.79	0.79	0.80
9	0.93	1.04	1.11	0.97	1.00	1.00
	Glucose					
1	1.86	2.14	1.88	2.01	2.13	2.05
2	1.86	2.00	—	1.99	2.15	—
3	2.24	2.08	2.45	2.19	2.03	2.34
4	2.22	2.35	—	2.48	2.52	—
5a	1.98	2.06	2.29	—	—	—
5b	1.80	1.87	1.86	1.94	1.96	1.93
6	1.92	1.98	2.01	1.91	1.92	1.96
7a	2.09	2.10	2.06	2.09	2.09	2.08
7b	2.02	2.20	1.85	2.07	2.07	1.98
8	1.94	1.96	1.98	2.00	2.00	2.02
9	2.45	2.36	2.75	2.38	2.33	2.34
	Sucrose					
1	4.39	4.19	4.10	3.90	3.93	3.88
2	4.32	4.30	—	4.45	4.75	—
3	4.90	4.87	4.63	5.21	5.06	5.03
4	4.00	4.09	—	4.76	4.80	—
5a	5.78	5.77	5.85	—	—	—
5b	5.68	5.35	5.75	5.46	6.39	5.60
6	4.73	4.57	4.63	4.81	4.78	4.78
7a	5.10	5.13	5.10	4.93	4.93	4.28
7b	4.56	4.73	4.31	4.57	4.57	4.48
8	4.90	5.02	4.94	4.94	4.91	4.94
9	4.87	4.75	4.44	4.67	4.78	4.54
	Maltose					
1	0.50	0.26	0.33	0.51	0.30	0.35
2	—	—	—	—	—	—
3[a]	0.72	0.55	1.45	0.52	0.50	1.19[b]
4	0.58	0.62	—	0.64	0.64	—
5a	—	—	—	—	—	—
5b	0.37	0.59	0.42	0.46	0.37	0.50
6	—	—	—	—	—	—
7a	—	—	—	—	—	—
7b	0.52	0.55	0.46	0.53	0.63	0.64
8	0.38	0.57	0.46	0.50	0.46	0.48
9	0.29	0.56	0.40	0.34	0.48	0.38

[a] Outlying variance (Cochran test).
[b] Outlier flagged for a replicate.

Table 6. Raw data (g/100 g) for cola drink (D2)

Laboratory	Area			Height		
	Fructose					
1	0.68	0.72	0.82	0.71	0.71	0.75
2	1.05	1.05	—	1.35[a]	1.45	—
3	0.76	0.79	0.72	0.82	0.78	0.79
4	0.82	0.74	—	0.90	0.86	—
5a	0.84	0.88	0.82	—	—	—
5b	0.64	0.70	0.65	0.66	0.73	0.66
6	0.45	0.59	0.57	0.54	0.63	0.62
7a	0.77	0.78	0.76	0.79	0.80	0.76
7b	0.75	0.77	0.74	0.74	0.73	0.78
8	0.74	0.69	0.67	0.68	0.67	0.66
9	0.64	0.75	0.82	0.75	0.79	0.77
	Glucose					
1[b]	1.75	1.95	2.26	1.98	2.10	2.23
2	1.86	1.94	—	2.10	2.17	—
3	2.58	2.73	2.54	2.29	2.35	2.43
4	2.22	2.13	—	2.43	2.43	—
5a	2.24	2.38	2.16	—	—	—
5b	2.02	2.07	2.0	2.09	2.15	2.11
6	1.93	2.09	2.06	1.97	2.07	2.01
7a	2.15	2.16	2.13	2.16	2.17	2.04
7b	2.01	1.99	2.04	2.00	2.08	2.04
8	2.15	2.09	2.06	2.10	2.10	2.09
9	2.12	2.41	2.25	2.21	2.39	2.22
	Sucrose					
1	5.66	5.80	5.62	5.21	5.28	5.23
2	6.08	5.85	—	6.23	5.97	—
3	6.21	6.34	5.94	6.37	6.40	6.59
4	5.54	5.56	—	6.38	6.43	—
5a	7.04	6.74	6.80	—	—	—
5b	6.82	6.54	6.77	6.64	6.54	6.67
6	6.04	6.02	5.96	6.11	6.13	6.08
7a	6.67	6.67	6.58	6.44	6.44	6.34
7b	5.84	6.41	5.73	5.84	6.00	5.85
8	6.22	6.29	6.21	6.25	6.21	6.20
9	6.60	6.25	5.98	6.00	6.35	6.03
	Maltose					
1	0.63	0.67	0.48	0.60	0.63	0.56
2	—	—	—	—	—	—
3	0.54	0.86	—	0.72	0.56	—
4	0.71	0.68	—	0.77	0.76	—
5a	—	—	—	—	—	—
5b	0.43	0.67	0.47	0.49	0.61	0.50
6	—	—	—	—	—	—
7a	—	—	—	—	—	—
7b	0.71	0.66	0.33	0.68	0.72	0.49
8	0.69	0.66	0.61	0.59	0.59	0.57
9	0.57	0.59	0.47	0.57	0.57	0.52

[a] Outlying mean (Grubbs test).
[b] Outlying variance (Cochran test).

"false" outliers: (*1*) A replicate can be an outlier for a single cell, although it is obviously within the range of all other cells; (*2*) data rounding may bias the computation of the ratio.

Thus, 2 complementary conditions were required before a replicate was rejected as an outlier: (i) it was significantly outlying at a 5% risk level; (ii) the laboratory variance was also detected as an outlier (Cochran test).

This preliminary procedure presents the advantage of discarding an isolated outlier quickly. Thus, a laboratory that would normally have been considered as an outlier for its large variance may be kept after a second test, as the variance computed with the remaining data is acceptable.

Results and Discussion

Results of the Interlaboratory Studies

For each food product, raw data are presented in Tables 5–16. Except for Study 2 on cereal products (maltose in product C1), no more than 2 of the 9 laboratories were dropped.

For each analyte, precision values, presented in Tables 17–21, were computed from either raw data or corrected data, after outlier rejection. The following parameters are given: M, reference value (global mean of all the laboratories); s_r, repeatability standard deviation (intralaboratory variance); r, repeatability at the 95% confidence level (2.83 s_r); RSD_r, repeatability relative standard deviation (100 s_r/M); s_R, reproducibility standard deviation (total variance); R, reproducibility at the 95% confidence level (2.83 s_R); and RSD_R, reproducibility relative standard deviation (100 s_R/M).

As expected, highest R values are observed for highest concentrations. The fitting of a linear regression model was not significant enough to assess any relationship between precision and concentration. Very different reproducibility values may be observable for close concentrations; for instance, for fructose and glucose, a concentration variation of 1 g/100 g may cause an 8-fold variation in reproducibility.

A linear relationship between R and M should mean that RSD_R is constant over the observed concentration range. Horwitz demonstrated that over a large concentration range, RSD_R is generally exponentially related to M, and proposed an empirical model for predicting this relationship (8). Figure 1 represents the 80 observed values of RSD_R as a function of the decimal logarithm of the concentration expressed in g/g (−log(M)). Two empirical Horwitz models were plotted from the following equations:

$$\text{Lower curve: } 2^{(1 - 0.5\log(M))}$$

$$\text{Upper curve: } 2^{(2 - 0.5\log(M))}$$

RSD_R values close to the lower curve indicate acceptable precision; values above the upper curve indicate questionable precision.

Most of the results shown in Figure 1 are considerably above Horwitz's curves. However, precision is acceptable for the majority of the results because the wide range of equipment and conditions used is very far from the standard reproducibility conditions. Another group of about 20 results (i.e., 25% of data) have an unacceptable precision; for some of them, the corresponding concentration levels are close to the detection limit, although this is not a complete explanation. Therefore, it was decided to determine which source of variation in the analytical procedure explains most of the variability and what role is played by the sugar or food product type in this variability.

Role of the Calculation Method

For Studies 1 and 3, soft drinks and confectionery, respectively, it was possible to compare performances obtained when the peak area or peak height was used to calculate the concen-

Table 7. Raw data (g/100 g) for orange juice 20% (D3)

Laboratory	Area			Height		
	Fructose					
1	2.76	2.60	2.80	2.41	2.40	2.46
2[a]	2.62	2.13	—	2.42	2.39	—
3	2.71	2.67	2.82	2.72	2.68	2.77
4	3.03	2.90	—	3.30	3.24	—
5a	2.91	2.91	2.82	—	—	—
5b	2.86	2.72	2.84	2.93	2.92	2.88
6	2.81	2.75	2.66	2.73	2.72	2.71
7a	2.62	2.57	2.65	2.42	2.63	2.68
7b	2.53	2.43	2.64	2.64	2.61	2.67
8	2.88	2.87	2.90	2.85	2.85	2.86
9	3.09	3.29	2.96	3.14	3.30	3.05
	Glucose					
1	3.23	2.75	3.22	2.69	2.60	2.70
2	2.52	2.70	—	2.46	2.50	—
3	2.72	2.91	2.89	2.77	2.85	2.88
4	3.36	3.10	—	3.49	3.39	—
5a	2.77	2.93	2.81	—	—	—
5b	2.78	2.73	2.77	2.88	2.89	2.88
6	2.76	2.66	2.62	2.89	2.85	2.80
7a	2.51	2.49	2.56	2.21	2.50	2.54
7b	2.54	2.38[b]	2.54	2.55	2.43	2.57
8	2.84	2.84	2.85	2.85	2.85	2.91
9	3.08	3.40[b]	3.09	3.07	3.37	3.08
	Sucrose					
1	3.27	3.41	3.06	2.96	3.03	2.89
2[c]	8.10	7.60	—	6.32[c]	6.02	—
3	3.29	3.48	3.66	3.48	3.49	3.67
4	3.26	3.19	—	3.65	3.62	—
5a	3.80	4.00	3.85	—	—	—
5b	4.19	4.03	4.12	4.17	4.18	4.17
6	3.73	3.65	3.75	3.75	3.70	3.73
7a	4.06	4.07	4.09	4.05	4.12	4.14
7b[a]	4.62	3.79	4.12	4.17	3.97	4.05
8	3.60	3.62	3.71	3.65	3.68	3.64
9	3.61	3.69	3.37	3.50	3.67	3.43

[a] Outlying variance (Cochran test).
[b] Outlier flagged for a replicate.
[c] Outlying mean (Grubbs test).

Table 8. Raw data (g/100 g) for pure orange juice (D4)

Laboratory	Area			Height		
	Fructose					
1	2.69	2.57	2.81	2.34	2.31	2.44
2	2.81	2.78	—	2.62	2.67	—
3	2.87	2.85	2.92	2.87	2.86	2.90
4	2.90	2.90	—	3.52	3.51	—
5a	2.90	3.17	3.07	—	—	—
5b	2.89	2.77	2.73	2.92	2.88	2.85
6[a]	4.43	4.26	4.39	4.43	4.26	4.39
7a	3.05	3.02	3.02	2.88	2.88	2.88
7b	2.51	2.55	2.57	2.74	2.78	2.74
8	2.81	2.78	2.82	2.74	2.76	2.74
9[b]	7.77	9.69	3.10	4.42[b]	5.24	3.53
	Glucose					
1	3.04	2.81	3.26	2.65	2.50	2.68
2	2.49	2.58	—	2.57	2.64	—
3	3.03	3.11	3.17	3.11	3.22	3.21
4	2.87	2.83	—	3.61	3.63	—
5a	2.71	2.79	3.05	—	—	—
5b	2.95	2.82	2.52	2.74	2.82	2.73
6	3.41	3.29	3.27	3.29	3.27	3.41
7a	2.74	2.74	2.79	2.67	2.66	2.68
7b	2.39	2.69	2.68	2.59	2.74	2.67
8	2.71	2.66	2.68	2.68	2.70	2.69
9[b]	4.71	5.77	3.11	3.77[b]	4.55	3.26
	Sucrose					
1	2.55	2.46	2.65	2.44	2.43	2.57
2	3.05	2.61	—	2.90	2.81	—
3	3.47	3.08	3.51	3.42	3.29	3.45
4	2.81	2.34	—	3.66	3.60	—
5a	2.96	2.69	3.08	—	—	—
5b	3.32	3.18	3.20	3.18	3.21	3.11
6	4.34	4.30	4.20	4.34	4.30	4.20
7a	2.86	2.82	2.86	2.89	2.86	2.89
7b	2.60	2.53	2.38	2.73	2.65	2.62
8	3.04	2.97	3.03	2.97	3.00	3.00
9	3.18	3.24	3.33	3.56	3.77	3.68
	Maltose					
1	0.19	0.10	0.11	0.15	0.16	0.15
2	—	—	—	—	—	—
3	0.60	0.41	0.50	0.44	0.51	0.42
4	0.45	0.44	—	0.62	0.62	—
5a	—	—	—	—	—	—
5b	0.17	0.16	0.15	0.19	0.19	0.18
6	—	—	—	—	—	—
7a	—	—	—	—	—	—
7b	0.31	0.41	0.27	0.38	0.32	0.40
8	0.35	0.29	0.29	0.32	0.30	0.31
9	—	—	—	—	—	—

[a] Outlying mean (Grubbs test).
[b] Outlying variance (Cochran test).

Table 9. Raw data (g/100 g) for orange syrup (D5)

Laboratory	Area			Height		
	Fructose					
1	21.07	21.24	21.24	18.60	18.85	19.19
2	17.30	16.60	—	19.15	18.05	—
3	18.76	17.95	18.06	18.59	18.06	17.75
4	22.57	22.09	—	25.48	26.11	—
5a	19.77	20.58	20.46	—	—	—
5b	22.56	22.38	24.14	19.80	19.85	20.08
6	28.64	29.50	29.30	33.17[a]	33.66	33.30
7a	19.85	20.50	20.14	20.28	20.59	20.45
7b	21.26	22.14	19.23	21.18	21.66	20.84
8	21.03	20.90	20.75	21.79	21.64	21.75
9	19.98	20.88	20.50	21.10	21.79	20.71
	Glucose					
1	25.16	27.72	29.17	24.57	25.76	26.53
2	25.50	25.57	—	21.95	23.80	—
3	23.89	23.81	26.93	21.94	23.39	22.46
4	30.95	28.45	—	34.22	33.29	—
5a	24.12	25.36	25.39	—	—	—
5b	28.01	28.25	30.68	23.60	23.62	24.11
6	28.06	28.54	28.46	36.09	36.52	36.26
7a	24.83	24.87	24.44	24.55	24.62	24.45
7b	22.35	30.20[b]	22.14	23.90	25.54	23.81
8	25.03	25.13	25.05	26.20	26.04	26.15
9	25.319	28.43	24.83	27.75	25.35	—
	Sucrose					
1	1.28	1.28	—	1.19	1.28	—
2	0.35	—	—	0.70	—	—
3[c]	1.74	0.97	0.58	1.60	0.73	0.81
4	0.61	0.99	—	0.76	0.91	—
5a	—	—	—	—	—	—
5b	0.65	0.65	0.93	0.63	0.61	1.11
6	0.62	0.69	0.59	1.04	1.10	1.00
7a	—	—	—	—	—	—
7b[c]	8.17	1.83	4.73	1.87[c]	1.52	1.97
8	1.22	0.79	0.70	0.85	0.73	0.78
9	C.79	0.86	0.68	0.96	0.98	0.88
	Maltose					
1	3.07	3.58	6.82	3.67	3.75	5.80
2	3.55	4.65	—	3.07	4.05	—
3	5.12	3.44	5.26	3.79	3.65	4.77
4	5.64	6.19	—	6.43	6.85	—
5a	5.66	5.91	6.71	—	—	—
5b	3.78	3.84	5.39	3.74	3.77	3.7
6	5.21	5.37	5.35	6.62	6.73	6.64
7a	5.79	5.72	5.66	5.14	5.10	5.22
7b	10.78	8.16	5.69	7.60	6.07	6.12
8	4.84	4.63	4.59	4.71	4.65	4.70
9	5.04	5.14	3.65	5.47	5.54	4.44

[a] Outlying mean (Grubbs test).
[b] Outlier flagged for a replicate.
[c] Outlying variance (Cochran test).

Table 10. Raw data (g/100 g) for crispbread (C1)

Laboratory	Area				Height			
	Sample preparation No. 1		Sample preparation No. 2		Sample preparation No. 1		Sample preparation No. 2	
				Fructose				
3	2.19	1.83	1.96	1.91	2.47[a]	2.48	2.63	2.77
6	2.13	2.02	2.10	2.15	2.11	2.02	2.09	2.10
9	2.406	2.228	2.409	2.298	2.256	2.195	2.301	2.215
10	—	—	—	—	2.45	2.45	2.33	2.42
11	2.27	2.25	2.20	2.17	2.29	2.28	2.23	2.22
12	2.90	2.90	2.70	2.90	—	—	—	—
13	1.73	1.73	—	—	—	—	—	—
14	2.03	2.02	2.11	2.09	2.01	1.97	2.01	2.00
				Glucose				
3	1.11	1.11	1.14	1.17	1.18	1.23	1.31	1.27
6	1.19	1.07	1.20	1.20	1.18	1.02	1.17	1.15
9	1.327	1.353	1.429	1.265	1.306	1.289	1.362	1.278
10	—	—	—	—	1.20	1.20	1.20	1.2
11	1.27	1.24	1.22	1.24	1.31	1.31	1.33	1.36
12	1.50	1.40	1.20	1.40	—	—	—	—
13[b]	0.66	0.55	—	—	—	—	—	—
14	0.95	1.12	1.13	1.02	1.05	1.14	1.14	1.07
				Maltose				
3[a]	3.69	2.85	3.60	3.20	3.12[a]	2.98	3.60	3.15
6	3.32	3.34	3.28	3.43	3.02	2.98	2.99	3.06
9	3.289	3.502	3.35	3.648	3.286	3.248	3.339	3.312
10	—	—	—	—	3.27	3.27	3.14	3.14
11	3.44	3.42	3.43	3.42	3.43	3.44	3.52	3.53
12[a]	3.20	4.10	2.90	3.50	—	—	—	—
13	3.76	3.68	—	—	—	—	—	—
14	3.59	3.75	3.84	3.75	3.40	3.46	3.53	3.52

[a] Outlying variance (Cochran test).
[b] Outlying mean (Grubbs test).

tration. All available data comparing both methods are displayed in Figure 2. The magnitude of some differences is surprisingly high; for instance, the results for glucose determination in orange syrup vary by a factor of 2 between calibrations using peak height or surface. Nevertheless, no systematic difference was observed. This raises the question: when and why does area give a better precision than height, and when are observed differences statistically significant?

(a) *Influence on repeatability.*—A significant difference between repeatability from peak area or peak surface can be demonstrated by comparing repeatability variances s^2_{ra} and s^2_{rh} computed from both data sets. It was established by comparing the ratio $F_{obs} = s^2_{rh}/s^2_{ra}$ to a Fisher variable in which degrees of freedom are equal to the numbers of results minus the number of laboratories for each data set.

Peak height repeatability is significantly better for the 18 analyses (55%) flagged by an arrow on Figure 2 (level = 5%). Repeatability using peak area is better for only 1 analysis (fructose in Sweet), where an unknown overlapping peak made peak identification difficult. The influence of the calculation method is particularly important for maltose (5 significant differences for 6 results).

(b) *Influence on reproducibility.*—The same study for repeatability was performed for reproducibility using the ratio $F_{obs} = s^2_{Ra}/s^2_{Rh}$, with both degrees of freedom equal to the number of results minus 1. In this case, peak height gives as many significant results as peak area; the peak area method improves fructose and glucose reproducibility for 3 identical products, whereas peak height is generally better for sucrose, maltose, and lactose. The chromatograms presented in Figure 3 illustrate the analytical complications encountered; area calculation gives better precision when peaks are poorly separated (fructose and glucose at high concentration levels), but baseline detection influences the height estimation. Conversely, height calculation leads to better precision for small, flat peaks, such as maltose or lactose, at low concentration levels.

Table 11. Raw data (g/100 g) for sweet cake (C2)

Laboratory	Area				Height			
	Sample preparation No. 1		Sample preparation No. 2		Sample preparation No. 1		Sample preparation No. 2	
				Fructose				
3	0.76	0.59	0.58	0.65	0.86	0.75	0.67	0.70
6	0.80	0.79	0.79	0.77	0.72	0.71	0.73	0.73
9	0.771	0.712	0.828	0.73	0.742	0.723	0.766	0.736
10	—	—	—	—	0.66	0.63	0.78	0.78
11	0.79	0.79	0.81	0.81	0.81	0.81	0.83	0.83
12	0.80	0.80	0.80	0.80	—	—	—	—
13[a]	0.48	0.71	—	—	—	—	—	—
14	0.63	0.70	0.59	0.73	0.66	0.69	0.64	0.74
				Glucose				
3	0.66	0.54	0.55	0.60	0.59[a]	0.64	0.61	0.58
6	0.68	0.69	0.67	0.67	0.61[a]	0.62	0.62	0.64
9	0.769	0.798	0.781	0.717	0.713	0.683	0.72	0.681
10	—	—	—	—	0.60	0.60	0.64	0.64
11	0.75	0.77	0.79	0.79	0.78	0.79	0.80	0.81
12[a]	0.60	0.90	0.80	0.60	—	—	—	—
13[b]	0.36	—	—	—	—	—	—	—
14	0.48	0.55	0.47	0.62	0.53[a]	0.58	0.53	0.66
				Sucrose				
3	18.63[c]	15.06	14.82	15.38	20.12	17.99	17.22	16.71
6	14.60	14.46	13.87	14.29	14.99	14.84	15.32	15.17
9	15.917	16.029	16.404	20.471[c]	17.848	16.233	17.152	17.056
10	—	—	—	—	15.68	14.67	16.85	16.77
11	16.55	16.51	17.52	17.68	16.91	16.99	18.47	18.52
12	15.20	15.20	15.90	15.00	—	—	—	—
13	16.17	15.97	—	—	—	—	—	—
14	16.05	15.64	15.95	15.34	16.12	16.00	16.04	15.77
				Maltose				
3	0.44	0.37	0.26	0.25	0.47	0.51	0.42	0.39
6	—	—	—	—	—	—	—	—
9	0.244	0.331	0.234	0.319	0.295	0.318	0.30	0.317
10	—	—	—	—	0.14	0.28	0.20	0.27
11	0.28	0.25	0.25	0.30	0.28	0.27	0.29	0.32
12	0.20	0.20	0.20	0.20	—	—	—	—
13[a]	0.62	0.95	—	—	—	—	—	—
14	0.77	0.61	0.63	0.76	0.84	0.60	0.75	0.84
				Lactose				
3	3.28	2.70	2.61	2.52	3.05	2.73	2.43	2.54
6	2.03	2.13	1.94	2.01	2.09	2.07	2.14	2.12
9	2.425	2.301	2.38	2.421	2.532	2.445	2.54	2.499
10	—	—	—	—	2.33	2.18	2.31	2.39
11	3.36	3.28	3.66	3.84	3.29	3.26	3.72	3.79
12	2.50	2.40	2.50	2.50	—	—	—	—
13	2.16	2.40	—	—	—	—	—	—
14	2.20	2.10	1.94	2.09	2.47	2.37	2.22	2.41

[a] Outlying variance (Cochran test).
[b] Outlying mean (Grubbs test).
[c] Outlier flagged for a replicate.

Table 12. Raw data (g/100 g) for chocolate cake (C3)

Laboratory	Area				Height			
	Sample preparation No. 1		Sample preparation No. 2		Sample preparation No. 1		Sample preparation No. 2	
				Fructose				
3	0.13	0.12	0.15	0.13	0.13	0.14	0.18	0.20
6[a]	0.11	0.22	0.23	0.26	0.13	0.18	0.19	0.18
9	0.219	0.168	0.251	0.204	0.183	0.17	0.191	0.182
10	—	—	—	—	0.12	0.12	0.17	0.11
11	0.27	0.26	0.24	0.24	0.27	0.27	0.25	0.25
12	0.30	0.30	0.30	0.30	—	—	—	—
13	—	—	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—
				Glucose				
3	0.11	0.11	0.10	0.11	0.16	0.11	0.12	0.17
6	0.04[b]	0.20	0.21	0.19	0.09[b]	0.17	0.16	0.16
9	0.241	0.147	0.306	0.23	0.222	0.167	0.242	0.192
10	—	—	—	—	0.16	0.16	0.17	0.17
11	0.43	0.44	0.40	0.41	0.44	0.44	0.42	0.43
12[a]	0.30	0.40	0.20	0.30	—	—	—	—
13	—	—	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—
				Sucrose				
3	30.43	30.41	30.50	29.38	31.50[a]	30.91	27.05	43.05
6	29.02	27.33	28.43	28.54	26.90	26.26	26.82	27.25
9	34.70	33.775	33.426	32.478	35.688	34.845	34.47	35.00
10	—	—	—	—	35.23	33.71	34.74	34.74
11	36.64	36.52	36.16	35.50	37.40	37.44	38.03	37.94
12	34.00	34.00	33.10	33.50	—	—	—	—
13	33.97	34.18	—	—	—	—	—	—
14	33.96	32.47	32.60	32.20	34.08	33.62	33.82	33.73
				Lactose				
3	0.10	0.15	0.21	—	0.27[b]	0.41	0.41	—
6	0.15	0.09	0.13	0.15	0.23	0.19	0.24	0.24
9	0.358	0.347	0.33	0.30	0.407	0.38	0.378	0.363
10	—	—	—	—	0.37	0.30	0.31	0.31
11	0.38	0.44	0.41	0.38	0.44	0.46	0.46	0.45
12	0.40	0.40	0.40	0.40	—	—	—	—
13[a]	0.32	0.66	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—

[a] Outlying variance (Cochran test).
[b] Outlier flagged for a replicate.

Role of the Laboratory Bias Components

Repeatability and reproducibility are 2 estimates of precision based on the intra- and interlaboratory variances obtained from the classical ISO model. This explains the total sum of squares SS_T as the sum of 2 sums of squares: (*1*) the squared deviations caused by the laboratory biases and generally denoted SS_L, and (*2*) the squared deviations from residual random effect, denoted SS_r.

ISO model:

$$X = m + L + \varepsilon$$

where m = general mean, L = laboratory bias, and ε = residual effect.

$$SS_T = SS_L + SS_r$$

The experimental design used in this study allowed for control of 2 components included in the laboratory bias: the sample

Table 13. Raw data (g/100 g) for nougat-filled chocolate (S1)

Laboratory	Area				Height			
	Sample preparation No. 1		Sample preparation No. 2		Sample preparation No. 1		Sample preparation No. 2	
				Fructose				
3[a]	4.84	3.83	4.05	3.48	1.46	1.66	1.67	2.18
4	2.69	2.43	2.21	2.35	2.62	2.67	2.24	2.26
6	2.62	2.68	2.69	2.66	2.76	2.77	2.70	2.66
9	2.64	2.67	2.69	2.63	2.685	2.665	2.74	2.70
11	1.71	1.70	—	—	1.68	1.65	—	—
12	2.05	2.05	2.05	2.05	2.10	2.05	2.10	2.05
15	2.35	2.13	2.15	2.25	2.36	2.23	2.19	2.32
16	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—
18	2.80	2.90	2.60	2.50	2.40	2.40	2.30	2.30
19	1.40	1.20	1.80	1.80	1.40	1.40	1.80	1.80
20	—	—	—	—	3.00	2.90	3.10	3.20
				Glucose				
3[a]	13.40	12.96	7.10	5.84	2.47	2.07	1.58	2.22
4	2.17	1.72	1.45	1.50	2.88	2.70	2.27	2.25
6	3.26	3.28	3.47	3.10	3.03	3.05	3.11	2.96
9	3.08	3.01	2.99	2.94	3.26	3.23	3.32	3.23
11	1.79	1.90	—	—	1.83	1.85	—	—
12	2.20	2.25	2.35	2.40	2.30	2.25	2.25	2.30
15	2.43	2.37	2.41	2.45	2.41	2.54	2.42	2.49
16	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—
18	3.30	4.10	3.00	2.80	3.00	3.00	2.90	2.80
19	1.60	1.60	2.80	2.10	1.90	1.70	2.40	2.20
20	—	—	—	—	4.50	4.50	4.60	4.90
				Sucrose				
3	43.04	43.33	43.52	43.82	37.73	44.10[b]	43.94	44.64
4	40.12	40.74	39.52	40.39	37.60[a]	37.63	34.34	34.69
6	39.29	39.49	40.93	40.75	40.45	39.67	40.27	39.74
9[a]	38.39	37.96	43.63	41.02	37.05[a]	36.935	40.72	40.20
11	42.48	42.61	—	—	42.50	42.69	—	—
12	40.40	40.65	40.75	40.25	41.05	41.10	40.90	40.60
15	42.50	42.38	42.60	42.90	42.65	42.64	42.44	42.92
16	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—
18	39.30	39.60	40.30	40.20	39.20	39.70	39.90	40.60
19	43.00	42.80	42.50	44.10	42.50	42.30	42.90	43.80
20	—	—	—	—	38.50	39.00	38.40	39.20
				Lactose				
3	4.33	3.87	7.16	6.49	3.88	4.93	6.13	6.03
4	10.33	11.39	7.17	9.27	8.78	8.00	6.57	6.96
6	5.99	6.04	6.16	6.25	6.26	6.22	6.25	6.21
9	5.87	5.92	6.445	6.13	6.22	6.25	6.94	6.60
11	6.31	6.47	—	—	6.63	6.86	—	—
12	8.25	7.50	7.30	7.25	7.35	7.05	6.95	6.85
15	5.50	5.73	5.63	5.48	4.72	6.40	6.58	5.71
16	—	—	—	—	—	—	—	—

Table 13. ***Continued***

Laboratory	Area				Height			
	Sample preparation No. 1		Sample preparation No. 2		Sample preparation No. 1		Sample preparation No. 2	
17	—	—	—	—	—	—	—	—
18	6.80	7.40	6.50	6.00	6.30	6.60	6.40	6.30
19	5.60	6.40	6.80	6.50	6.20	6.60	6.70	6.80
20	—	—	—	—	5.80	6.10	—	6.10

[a] Outlying variance (Cochran test).
[b] Outlier flagged for a replicate.

preparation effect and the calculation method effect. The calculation method effect is fixed and takes 2 values: area and height. The sample preparation effect is random; the levels were the extraction number (1 and 2). Thus, the following mixed model can be used to separate the laboratory bias from other effects:

Extended model:

$$X = m + L + c + E + cL + EL + \varepsilon$$

where m = general mean, L = "pure" laboratory bias, c = calculation method effect, E = sample preparation effect, cL = interaction laboratory * calculation, EL = interaction laboratory * sample preparation, and ε = random effect.

The interaction between sample preparation and calculation method was ignored, and the remaining effects were estimated by means of a 3-way analysis of variance. Finally, the total sum of squares was broken down into the following equation:

$$SS_T = SS_L + SS_c + SS_E + SS_{cL} + SS_{EL} + {}^*SS_{cE} + {}^*SS_{LcE} + SS_r$$

* negligible

Without any assumption about data distribution, it is possible to express SS_L, SS_c, SS_E, SS_{cL}, SS_{EL}, and SS_r as a percentage of SS_T. Figure 4 represents the 45 available results on a cumulative bar chart. Each bar gives, for each analysis, the portion of variability explained by these 6 sums of squares. The pattern is observed to be quite different from one analysis to another, and several conclusions can be drawn.

The SS_{cL} and SS_{EL} sums of square are greater than the main factorial sums SS_c and SS_E. This demonstrates that the sample preparation step and the calculation method depend upon the laboratory. A nested model would even be better adapted, as the sums of squares explaining the calculation method and the sample preparation are expressed as:

$$SS_{calc} = SS_c + SS_{cL}$$

$$SS_{prep} = SS_E + SS_{EL}$$

Moreover, 4 groups of pattern are visible in Figure 4. Group I, in which 100 SS_L/SS_T is above 66%, has the most expected pattern, when no specific problem occurs and the main variability source comes from the differences between apparatus, standardization curves, operators, etc. Group II contains interlaboratory analyses in which sample preparation was particularly important; it groups heterogeneous products, such as nougat-filled chocolate and chocolate bar, that contain sticky ingredients preventing a good mixing, and sweetened biscuit and "Rocher" cream, which are sensitive to sucrose inversion when heating time is poorly controlled. Group III contains the 2 foods for which the calculation method was particularly important, the orange syrup and nougat-filled chocolate. The last group, IV, groups analytes having a low level of concentration, close to the detection limit, which were analyzed by only a few laboratories. The maltose precision is often very poor because of its low response factor and its proximity to the sucrose peak.

Altogether, for 45 results, the average 100 SS_{calc}/SS_T is equal to 10.5%. This percentage is very near to that obtained for sample preparation. Therefore, the variability introduced by the calculation method has the same importance as sample preparation.

Conclusion

This interlaboratory study demonstrated that LC can present an acceptable precision for most mono- and disaccharide analyses in foods, even when laboratories have variable equipment and sample matrixes are complex. For a concentration range varying from 1 to 30 g/100 g, the repeatability is generally about 5% and the reproducibility about 10%.

Besides these satisfactory results, 6 different sources of variation were characterized as the result of a more complex experimental design. This gives some clues for explaining the analytical difficulties encountered and indicates how they can be solved: by improvement of peak separation for analyte, such as maltose, by control of heat treatment for food products with high sucrose concentrations, etc.

The most striking result, however, consists in the impact of the method for calculating concentration on the final performance: the choice between peak area and peak height must be strictly considered. This must be emphasized, as it is generally neglected in many standards and official methods. Therefore, we consider that all methods based on chromatography that use computerized integrators must be checked, and guidelines must be issued to define which computation method is more suitable.

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Table 14. Raw data (g/100 g) for sweet (S2)

Laboratory	Area				Height			
	Sample preparation No. 1		Sample preparation No. 2		Sample preparation No. 1		Sample preparation No. 2	
				Fructose				
3	—	—	—	—	—	—	—	—
4[a]	5.15	4.77	3.68	3.52	5.41[a]	5.19	4.25	4.13
6	0.40	0.38	0.40	0.38	0.46	0.47	0.43	0.45
9	0.47	0.58	0.48	0.62	0.47	0.51	0.52	0.55
11	0.60	0.59	—	—	0.63	0.60	—	—
12	0.75	0.75	0.60	0.60	0.80	0.80	0.65	0.65
15	0.85	0.90	0.90	0.91	0.44	0.59	0.60	0.52
16	0.66	0.60	0.63	0.57	0.94	0.58	0.82	0.72
17	0.52	0.54	0.57	0.51	0.47	0.44	0.45	0.49
18[a]	0.90	0.50	0.40	0.90	0.80	0.50	0.50	0.80
19	0.40	0.40	0.40	—	0.30	0.40	0.30	0.30
20	—	—	—	—	7.30[a]	7.40	14.00	14.20
				Glucose				
3	2.75	2.58	2.17	2.21	2.88	2.93	2.46	2.40
4[a]	7.65	7.34	4.51	4.72	7.03[b]	6.79	6.08	6.12
6	2.87	2.96	2.84	2.64	3.05	3.14	3.08	2.96
9	3.23	3.11	3.23	3.16	3.44	3.36	3.50	3.455
11	3.23	3.04	—	—	3.24	3.18	—	—
12	3.50	3.65	3.65	3.70	3.55	3.55	3.30	3.45
15	3.90	3.92	3.63	3.23	2.99	3.04	3.26	3.31
16	3.92	4.39	4.36	4.27	4.53	4.77	5.10	4.86
17	3.06	3.03	3.37	3.00	3.14	3.05	3.20	3.06
18	3.20	2.80	3.00	4.10	3.30	3.00	3.00	3.50
19	3.70	4.00	3.20	2.50	3.30	3.50	3.20	2.70
20	—	—	—	—	10.80[a]	10.60	17.30	17.60
				Sucrose				
3	49.57	49.43	49.63	48.65	45.12	45.87	45.96	44.05
4	42.19	43.04	43.64	43.22	40.38	40.58	41.34	41.43
6	49.53	51.02	50.01	51.03	53.42	54.41	54.38	54.50
9	54.65	56.62	56.04	55.62	52.17	51.58	51.665	52.34
11	50.97	50.65	—	—	52.36	52.06	—	—
12	48.15	47.20	48.00	47.60	51.10	50.00	50.40	49.95
15	54.12	54.50	52.30	54.70	54.77	54.39	53.02	53.08
16[b]	76.13	75.26	79.61	77.87	77.16[b]	77.39	79.20	78.73
17	53.08	52.22	52.69	52.97	51.23	50.87	51.62	51.84
18	51.40	54.90	56.70	53.50	51.60	53.50	53.00	52.70
19	54.70	54.80	54.50	54.70	51.70	53.70	54.50	53.10
20	—	—	—	—	38.60[a]	38.50	25.40	25.30
				Maltose				
3	8.80	9.10	9.25	9.33	10.17	10.82	9.70	10.04
4[a]	10.16	14.36	11.33	12.94	9.20[a]	10.88	9.47	9.73
6	8.81	8.94	8.80	9.16	8.49	8.68	8.66	8.68
9	8.54	7.81	8.74	7.83	8.72	8.34	8.75	8.29
11	10.60	10.43	—	—	9.42	9.33	—	—
12	8.20	8.20	7.95	8.20	8.70	8.70	8.55	8.80
15	8.22	8.38	7.93	9.06	8.46	8.29	7.96	8.58
16	12.04	12.17	12.24	11.78	12.23	12.06	12.64	12.41

Table 14. ***Continued***

	Area				Height			
Laboratory	Sample preparation No. 1		Sample preparation No. 2		Sample preparation No. 1		Sample preparation No. 2	
17	10.18	10.02	10.12	9.84	8.73	8.92	8.91	8.83
18	9.20	9.50	9.80	9.80	8.90	9.00	9.00	9.10
19	6.20	5.50	5.80	7.20	6.90	7.10	7.10	7.10
20	—	—	—	—	10.00	9.80	9.40	9.30

[a] Outlying variance (Cochran test).
[b] Outlying mean (Grubbs test).

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Table 15. Raw data (g/100 g) for Rocher cream (S3)

	Area				Height			
Laboratory	Sample preparation No. 1		Sample preparation No. 2		Sample preparation No. 1		Sample preparation No. 2	
				Sucrose				
3[a]	35.61	36.15	40.58	48.65	40.61[a]	41.78	41.15	44.05
4	42.05	40.84	40.76	42.35	38.55	38.12	38.44	38.44
6	39.04	39.76	39.80	39.91	40.43	41.52	41.92	41.76
9	39.05	37.60	37.47	38.54	41.46	40.90	41.13	41.29
11	41.91	40.73	—	—	41.45	41.79	—	—
12	36.85	37.65	38.40	37.80	38.50	38.75	39.05	39.00
15	42.21	41.89	42.04	42.30	42.09	41.46	42.36	42.08
16	42.65	41.74	41.35	41.43	42.57	42.24	42.19	42.20
17	41.92	41.97	41.36	41.30	41.67	41.81	40.88	41.17
18	46.60[b]	42.40	42.40	42.20	42.90[a]	41.20	40.20	40.70
19	39.20	39.70	38.10	38.70	39.80	39.90	39.20	39.20
20	—	—	—	—	40.90	41.10	40.40	40.80
				Lactose				
3	1.61	1.23	1.11	1.30	2.60[a]	2.10	1.60	0.60
4	3.75	3.71	2.81	2.45	3.67	3.21	3.26	2.65
6	2.43	2.47	2.36	2.26	2.31	2.36	2.36	2.32
9	2.75	2.24	2.84	2.28	2.54	2.34	2.60	2.54
11	1.87	1.82	—	—	2.22	2.28	—	—
12	2.40	2.55	2.45	2.20	2.40	2.55	2.50	2.30
15	2.38	2.70	2.68	2.72	3.15	3.01	2.82	2.92
16	2.32	2.34	2.43	2.22	2.62	2.51	2.33	2.28
17	2.16	2.12	2.29	2.17	2.35	2.31	2.42	2.21
18	3.10	3.10	2.80	2.60	2.60	2.60	2.50	2.40
19	2.20	2.70	3.30	2.00	2.20	2.90	3.10	2.60
20	—	—	—	—	2.90	3.10	2.80	2.90

[a] Outlying variance (Cochran test).
[b] Outlier flagged for a replicate.

Table 16. Raw data (g/100 g) for chocolate bar (S4)

Laboratory	Area				Height			
	Sample preparation No. 1		Sample preparation No. 2		Sample preparation No. 1		Sample preparation No. 2	
				Glucose				
2	—	—	—	—	7.40	7.50	7.40	7.40
3[a]	7.43	8.44	9.72	8.21	6.77	6.61	7.52	7.91
4	6.01[b]	6.87	6.80	7.00	6.46	6.83	6.93	6.79
6	8.23	8.15	7.93	7.89	7.94	7.86	7.76	7.78
9	8.26	8.22	8.05	8.57	7.96	8.03	8.16	8.25
11	7.21	7.22	—	—	7.38	7.42	—	—
12[a]	7.70	8.10	8.10	8.60	7.90	7.90	7.50	7.75
15	8.08	8.30	8.10	8.50	8.57	8.85	8.52	8.01
16	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—
18	7.90	7.20	8.00	7.40	6.60	6.40	7.60	7.60
19	8.50	7.20	9.50	10.40	7.30	7.10	8.60	8.70
				Sucrose				
2	—	—	—	—	36.80	37.30	37.80	38.10
3	36.42	36.46	36.34	36.47	36.15	33.29	34.08	34.40
4	35.02	35.66	34.56	35.60	31.35[b]	34.83	34.77	34.65
6	37.22	37.28	36.92	36.74	38.66	37.86	37.77	37.86
9[a]	34.27	35.71	35.33	38.89	36.96	37.38	36.96	38.14
11	37.20	36.98	—	—	37.57	37.69	—	—
12	36.05	34.55	35.95	34.85	37.45	36.70	36.75	36.30
15	37.90	38.15	38.21	38.04	37.28	38.18	39.76	38.22
16	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—
18	37.20	39.2	37.00	39.70	37.10	37.80	36.90	38.30
19	38.40	39.0	37.50	36.70	38.30	39.30	37.60	37.50
				Maltose				
2	—	—	—	—	8.10	8.50	8.10	8.30
3	7.81	9.05	7.29	8.42	9.05	8.42	9.90	9.00
4[a]	8.52	9.26	11.37	12.20	7.65[a]	8.32	10.15	10.35
6	7.27	7.17	6.90	6.97	7.52	7.41	7.14	7.26
9	7.16	7.49	7.66	7.55	7.03	7.29	7.43	7.34
11	7.78	7.71	—	—	7.63	7.66	—	—
12	7.30	7.05	6.75	6.90	7.75	7.50	7.15	7.30
15	7.62	7.48	6.26	7.34	7.16	7.73	7.71	7.05
16	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—
18	7.30	7.50	7.70	7.60	7.50	7.50	7.50	7.50
19	6.20	6.10	7.10	7.10	6.00	6.10	7.20	7.10
				Lactose				
2	—	—	—	—	7.30	7.70	7.40	7.40
3	6.82	7.72	7.14	7.27	6.62	6.10	7.53	7.06
4	9.48	10.40	14.75[b]	10.07	7.87[a]	8.05	11.75	10.25
6	7.63	7.30	7.48	7.42	7.79	7.54	7.63	7.65
9	8.53	9.15	9.08	8.93	8.46	8.50	8.79	8.84
11	8.47	7.95	—	—	8.2	8.19	—	—
12	8.20	7.80	7.65	7.75	8.25	8.00	7.85	7.90
15	8.05	8.20	7.53	7.89	7.84	7.95	8.90	8.10

Table 16. ***Continued***

Laboratory	Area				Height			
	Sample preparation No. 1		Sample preparation No. 2		Sample preparation No. 1		Sample preparation No. 2	
16	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—
18	8.00	8.40	8.40	8.40	7.90	8.10	8.00	8.10
19	8.20	7.90	6.80	7.50	8.10	7.80	7.00	7.60

[a] Outlying variance (Cochran test).
[b] Outlier flagged for a replicate.

Table 17. Statistical results for fructose

Product	Unit	Area									
		D1	D2	D3	D4	D5	C1	C2	C3	S1	S2
Total laboratories		11	11	11	11	11	—	—	—	9	10
Rejected laboratories		0	0	1	2	0	—	—	—	1	2
Mean (raw data)	g/100 g	0.893	0.745	2.770	3.349	21.317	—	—	—	2.490	0.994
Mean (without outliers)	g/100 g	—	—	2.796	2.828	—	—	—	—	2.282	0.585
s_r (raw data)	g/100 g	0.073	0.051	0.113	1.049	0.638	—	—	—	0.247	0.285
r (raw data)	g/100 g	0.206	0.143	0.321	2.969	1.805	—	—	—	0.699	0.806
RSD_r (raw data)	%	8.158	6.780	4.098	31.324	2.993	—	—	—	9.921	28.652
s_R (raw data)	g/100 g	0.153	0.122	0.214	1.536	3.106	—	—	—	0.759	1.241
R (raw data)	g/100 g	0.434	0.345	0.607	4.347	8.789	—	—	—	2.148	3.511
RSD_R (raw data)	%	17.166	16.376	7.738	45.871	14.568	—	—	—	30.480	124.82
s_r (without outliers)	g/100 g	0.073	0.051	0.087	0.072	0.638	—	—	—	0.155	0.048
r (without outliers)	g/100 g	0.206	0.143	0.246	0.203	1.805	—	—	—	0.438	0.136
RSD_r (without outliers)	%	8.158	6.780	3.103	2.532	2.993	—	—	—	6.790	8.194
s_R (without outliers)	g/100 g	0.153	0.122	0.182	0.171	3.106	—	—	—	0.455	0.169
R (without outliers)	g/100 g	0.434	0.345	0.514	0.483	8.789	—	—	—	1.288	0.479
RSD_R (without outliers)	%	17.166	16.376	6.500	6.039	14.568	—	—	—	19.954	28.959
		Height									
Total laboratories		10	10	10	10	10	8	8	6	10	11
Rejected laboratories		0	0	0	1	1	0	1	0	0	2
Mean (raw data)	g/100 g	0.933	0.779	2.767	3.132	21.906	2.305	0.736	0.196	2.294	1.925
Mean (without outliers)	g/100 g	—	—	—	2.987	20.583	—	0.728	—	—	0.557
s_r (raw data)	g/100 g	0.034	0.033	0.066	0.281	0.364	0.073	0.058	0.017	0.157	1.232
r (raw data)	g/100 g	0.096	0.092	0.186	0.794	1.029	0.206	0.163	0.049	0.445	3.487
RSD_r (raw data)	%	3.640	4.174	2.376	8.951	1.660	3.157	7.843	8.934	6.860	64.023
s_R (raw data)	g/100 g	0.185	0.195	0.273	0.754	4.560	0.334	0.079	0.071	0.500	3.431
R (raw data)	g/100 g	0.523	0.552	0.772	2.135	12.905	0.946	0.224	0.201	1.415	9.710
RSD_R (raw data)	%	19.817	25.047	9.854	24.086	20.816	14.508	10.727	36.377	21.799	178.27
s_r (without outliers)	g/100 g	0.034	0.033	0.066	0.043	0.375	0.073	0.047	0.017	0.157	0.092
r (without outliers)	g/100 g	0.096	0.092	0.186	0.121	1.060	0.206	0.134	0.049	0.445	0.261
RSD_r (without outliers)	%	3.640	4.174	2.376	1.431	1.819	3.157	6.332	8.934	6.860	16.540
s_R (without outliers)	g/100 g	0.185	0.195	0.273	0.595	2.088	0.334	0.064	0.071	0.500	0.165
R (without outliers)	g/100 g	0.523	0.552	0.772	1.684	5.908	0.946	0.180	0.201	1.415	0.467
RSD_R (without outliers)	%	19.817	25.047	9.854	9.922	10.143	14.508	8.504	36.377	21.799	29.594

Table 18. Statistical results for glucose

Product	Unit	D1	D2	D3	D4	D5	C1	C2	C3	S1	S2	S4
						Area						
Total laboratories		11	11	11	11	11	—	—	—	9	11	9
Rejected laboratories		0	1	0	1	1	—	—	—	1	1	2
Mean (raw data)	g/100 g	2.081	2.141	2.819	3.012	26.304	—	—	—	3.386	3.555	7.994
Mean (without outliers)	g/100 g	—	2.157	—	2.855	26.178	—	—	—	2.527	3.292	7.784
s_r (raw data)	g/100 g	0.128	0.110	0.127	0.433	1.867	—	—	—	1.390	0.610	0.638
r (raw data)	g/100 g	0.361	0.310	0.358	1.224	5.284	—	—	—	3.934	1.726	1.805
RSD_r (raw data)	%	6.128	5.122	4.487	14.363	7.098	—	—	—	41.051	17.152	7.978
s_R (raw data)	g/100 g	0.222	0.213	0.262	0.662	2.342	—	—	—	2.829	1.108	0.865
R (raw data)	g/100 g	0.628	0.604	0.742	1.874	6.627	—	—	—	8.007	3.135	2.448
RSD_R (raw data)	%	10.660	9.963	9.305	21.986	8.903	—	—	—	83.566	31.154	10.820
s_r (without outliers)	g/100 g	—	—	—	—	—	—	—	—	0.329	0.336	0.307
r (without outliers)	g/100 g	0.361	0.225	0.358	0.384	3.524	—	—	—	0.932	0.951	0.870
RSD_r (without outliers)	%	6.128	3.693	4.487	4.750	4.757	—	—	—	13.026	10.209	3.950
s_R (without outliers)	g/100 g	0.222	0.207	0.262	0.266	2.291	—	—	—	0.692	0.581	0.665
R (without outliers)	g/100 g	0.628	0.587	0.742	0.753	6.484	—	—	—	1.957	1.643	1.882
RSD_R (without outliers)	%	10.660	9.621	9.305	9.315	8.753	—	—	—	27.360	17.637	8.545
						Height						
Total laboratories		10	10	10	10	10	8	8	6	10	12	10
Rejected laboratories		0	0	0	1	0	1	1	1	0	2	0
Mean (raw data)	g/100 g	2.102	2.160	2.804	2.980	26.519	1 199	0.653	0.226	2.754	4.545	7.604
Mean (without outliers)	g/100 g	—	—	—	2.878	—	1 241	0.641	0.218	—	3.335	—
s_r (raw data)	g/100 g	0.064	0.067	0.092	0.033	0.734	0.065	0.064	0.039	0.205	1.181	0.430
r (raw data)	g/100 g	0.181	0.191	0.260	0.092	2.077	0.183	0.180	0.111	0.580	3.341	1.217
RSD_r (raw data)	%	3.051	3.124	3.280	1.044	2.768	5.391	9.730	17.398	7.437	25.980	5.654
s_R (raw data)	g/100 g	0.177	0.141	0.302	0.746	4.488	0.204	0.111	0.125	0.829	3.409	0.651
R (raw data)	g/100 g	0.501	0.398	0.855	2.111	12.701	0.578	0.315	0.355	2.345	9.648	1.843
RSD_R (raw data)	%	8.414	6.512	10.771	24.022	16.923	17.046	17.039	55.505	30.087	75.011	8.564
s_r (without outliers)	g/100 g	0.064	0.067	0.092	0.218	0.734	0.064	0.031	0.017	0.205	0.197	0.430
r (without outliers)	g/100 g	0.181	0.191	0.260	0.616	2.077	0.181	0.087	0.047	0.580	0.557	1.217
RSD_r (without outliers)	%	3.051	3.124	3.280	7.301	2.768	5.150	4.821	7.559	7.437	5.899	5.654
s_R (without outliers)	g/100 g	0.177	0.141	0.302	0.488	4.488	0.117	0.104	0.130	0.829	0.605	0.651
R (without outliers)	g/100 g	0.501	0.398	0.855	1.380	12.701	0.332	0.294	0.368	2.345	1.711	1.843
RSD_R (without outliers)	%	8.414	6.512	10.771	16.365	16.923	9.438	16.192	59.824	30.087	18.133	8.564

Table 19. Statistical results for sucrose

		Area										
Product	Unit	D1	D2	D3	D4	D5	C2	C3	S1	S2	S3	S4
Total laboratories		11	11	11	11	10	—	—	9	11	11	9
Rejected laboratories		0	0	2	0	3	—	—	1	1	1	1
Mean (raw data)	g/100 g	4.835	6.218	3.982	3.053	1.877	—	—	41.331	53.741	40.547	36.808
Mean (without outliers)	g/100 g	—	—	3.674	—	0.799	—	—	41.475	51.269	40.415	36.909
s_r (raw data)	g/100 g	0.136	0.182	0.184	0.152	1.132	—	—	1.028	1.083	2.062	0.969
r (raw data)	g/100 g	0.386	0.516	0.520	0.429	3.203	—	—	2.910	3.066	5.835	2.742
RSD_r (raw data)	%	2.822	2.932	4.618	4.965	60.294	—	—	2.488	2.016	5.085	2.632
s_R (raw data)	g/100 g	0.542	0.427	1.107	0.520	2.267	—	—	1.729	8.882	2.549	1.441
R (raw data)	g/100 g	1.534	1.207	3.133	1.471	6.416	—	—	4.894	25.136	7.213	4.078
RSD_R (raw data)	%	11.212	6.862	27.798	17.030	120.778	—	—	4.184	16.528	6.286	3.915
s_r (without outliers)	g/100 g	0.136	0.182	0.136	0.152	0.174	—	—	0.510	0.949	0.581	0.725
r (without outliers)	g/100 g	0.386	0.516	0.386	0.429	0.492	—	—	1.443	2.687	1.643	2.052
RSD_r (without outliers)	%	2.822	2.932	3.616	4.965	21.759	—	—	1.230	1.852	1.437	1.964
s_R (without outliers)	g/100 g	0.542	0.427	0.445	0.520	0.263	—	—	1.593	4.046	1.839	1.373
R (without outliers)	g/100 g	1.534	1.207	1.259	1.471	0.744	—	—	4.509	11.451	5.204	3.885
RSD_R (without outliers)	%	11.212	6.862	11.778	17.030	32.895	—	—	3.841	7.892	4.550	3.719
		Height										
Total laboratories		10	10	10	10	9	8	8	10	12	12	10
Rejected laboratories		0	0	1	0	0	0	0	2	2	2	2
Mean (raw data)	g/100 g	4.794	6.153	3.881	3.190	1.051	16.429	33.173	40.400	51.304	40.850	36.943
Mean (without outliers)	g/100 g	—	—	3.711	—	—	—	—	41.321	50.518	40.698	37.677
s_r (raw data)	g/100 g	0.216	0.096	0.085	0.065	0.247	0.807	0.447	1.430	2.374	0.657	0.900
r (raw data)	g/100 g	0.611	0.271	0.240	0.183	0.699	2.285	1.266	4.048	6.718	1.858	2.546
RSD_r (raw data)	%	4.504	1.556	2.187	2.024	23.485	4.915	1.348	3.540	4.627	1.607	2.436
s_R (raw data)	g/100 g	0.519	0.398	0.753	0.549	0.381	1.309	3.442	2.569	11.131	1.411	1.753
R (raw data)	g/100 g	1.469	1.127	2.131	1.555	1.077	3.705	9.740	7.271	31.501	3.994	4.962
RSD_R (raw data)	%	10.823	6.472	19.406	17.224	36.191	7.969	10.375	6.359	21.697	3.455	4.746
s_r (without outliers)	g/100 g	0.216	0.096	0.071	0.065	0.247	0.807	0.447	0.425	0.889	0.362	0.661
r (without outliers)	g/100 g	0.611	0.271	0.202	0.183	0.699	2.285	1.266	1.202	2.517	1.024	1.871
RSD_r (without outliers)	%	4.504	1.556	1.924	2.024	23.485	4.915	1.348	1.028	1.753	0.889	1.754
s_R (without outliers)	g/100 g	0.519	0.398	0.380	0.549	0.381	1.309	3.442	1.895	6.081	1.410	0.769
R (without outliers)	g/100 g	1.469	1.127	1.075	1.555	1.077	3.705	9.740	5.362	17.209	3.991	2.175
RSD_R (without outliers)	%	10.823	6.472	10.232	17.224	36.191	7.969	10.375	4.585	11.987	3.465	2.040

Table 20. Statistical results for maltose

Product	Unit	Area							
		D1	D2	D4	D5	C1	C2	S2	S4
Total laboratories		7	7	6	10	—	—	11	9
Rejected laboratories		1	0	0	0	—	—	1	1
Mean (raw data)	g/100 g	0.531	0.627	0.305	5.193	—	—	9.344	7.673
Mean (without outliers)	g/100 g	0.468	—	—	—	—	—	9.044	7.318
s_r (raw data)	g/100 g	0.204	0.154	0.055	1.195	—	—	0.670	0.729
r (raw data)	g/100 g	0.577	0.437	0.156	3.383	—	—	1.896	2.063
RSD_r (raw data)	%	38.450	24.672	18.029	23.019	—	—	7.168	9.499
s_R (raw data)	g/100 g	0.245	0.168	0.151	1.553	—	—	1.882	1.287
R (raw data)	g/100 g	0.693	0.475	0.428	4.395	—	—	5.327	3.643
RSD_R (raw data)	%	46.164	26.803	49.580	29.906	—	—	20.142	16.777
s_r (without outliers)	g/100 g	0.102	0.154	0.055	1.195	—	—	0.367	0.441
r (without outliers)	g/100 g	0.289	0.437	0.156	3.383	—	—	1.039	1.248
RSD_r (without outliers)	%	21.840	24.672	18.029	23.019	—	—	4.060	6.028
s_R (without outliers)	g/100 g	0.112	0.168	0.151	1.553	—	—	1.622	0.610
R (without outliers)	g/100 g	0.317	0.475	0.428	4.395	—	—	4.591	1.725
RSD_R (without outliers)	%	23.932	26.803	49.580	29.906	—	—	17.936	8.328
		Height							
Total laboratories		7	7	6	10	8	7	12	10
Rejected laboratories		0	0	0	0	2	1	1	1
Mean (raw data)	g/100 g	0.516	0.608	0.333	5.057	3.322	0.403	9.192	7.771
Mean (without outliers)	g/100 g	—	—	—	—	3.323	0.371	9.132	7.613
s_r (raw data)	g/100 g	0.161	0.067	0.027	0.605	0.218	0.078	0.313	0.554
r (raw data)	g/100 g	0.456	0.191	0.076	1.712	0.616	0.221	0.887	1.567
RSD_r (raw data)	%	31.210	11.134	8.029	11.964	6.557	19.411	3.410	7.123
s_R (raw data)	g/100 g	0.187	0.087	0.160	1.263	0.269	0.241	1.335	0.960
R (raw data)	g/100 g	0.528	0.246	0.454	3.575	0.762	0.681	3.777	2.716
RSD_R (raw data)	%	36.134	14.331	48.225	24.986	8.109	59.781	14.518	12.349
s_r (without outliers)	g/100 g	0.161	0.067	0.027	0.605	0.050	0.059	0.234	0.358
r (without outliers)	g/100 g	0.456	0.191	0.076	1.712	0.141	0.166	0.663	1.013
RSD_r (without outliers)	%	31.210	11.134	8.029	11.964	1.498	15.780	2.566	4.703
s_R (without outliers)	g/100 g	0.187	0.087	0.160	1.263	0.226	0.214	1.372	0.783
R (without outliers)	g/100 g	0.528	0.246	0.454	3.575	0.640	0.607	3.883	2.215
RSD_R (without outliers)	%	36.134	14.331	48.225	24.986	6.810	57.831	15.025	10.281

Table 21. Statistical results for lactose

		Area				
Product	Unit	C2	C3	S1	S3	S4
Total laboratories		—	—	9	11	9
Rejected laboratories		—	—	0	0	1
Mean (raw data)	g/100 g	—	—	6.654	2.410	8.302
Mean (without outliers)	g/100 g	—	—	—	—	7.919
s_r (raw data)	g/100 g	—	—	0.900	0.316	0.894
r (raw data)	g/100 g	—	—	2.547	0.895	2.529
RSD_r (raw data)	%	—	—	13.526	13.123	10.763
s_R (raw data)	g/100 g	—	—	1.501	0.565	1.446
R (raw data)	g/100 g	—	—	4.249	1.598	4.093
RSD_R (raw data)	%	—	—	22.565	23.426	17.421
s_r (without outliers)	g/100 g	—	—	0.900	0.316	0.336
r (without outliers)	g/100 g	—	—	2.547	0.895	0.951
RSD_r (without outliers)	%	—	—	13.526	13.123	4.243
s_R (without outliers)	g/100 g	—	—	1.501	0.565	0.621
R (without outliers)	g/100 g	—	—	4.249	1.598	1.757
RSD_R (without outliers)	%	—	—	22.565	23.426	7.840
		Height				
Total laboratories		8	7	10	12	10
Rejected laboratories		0	1	0	1	1
Mean (raw data)	g/100 g	2.546	0.368	6.412	2.540	8.001
Mean (without outliers)	g/100 g	—	0.361	—	2.618	7.826
s_r (raw data)	g/100 g	0.159	0.065	0.589	0.320	0.687
r (raw data)	g/100 g	0.450	0.185	1.666	0.906	1.943
RSD_r (raw data)	%	6.249	17.768	9.180	12.606	8.582
s_R (raw data)	g/100 g	0.464	0.101	0.835	0.478	0.942
R (raw data)	g/100 g	1.314	0.287	2.364	1.354	2.666
RSD_R (raw data)	%	18.232	27.551	13.029	18.841	11.775
s_r (without outliers)	g/100 g	0.159	0.019	0.589	0.205	0.336
r (without outliers)	g/100 g	0.450	0.055	1.666	0.579	0.950
RSD_r (without outliers)	%	6.249	5.385	9.180	7.818	4.290
s_R (without outliers)	g/100 g	0.464	0.085	0.835	0.352	0.608
R (without outliers)	g/100 g	1.314	0.240	2.364	0.996	1.721
RSD_R (without outliers)	%	18.232	23.499	13.029	13.445	7.771

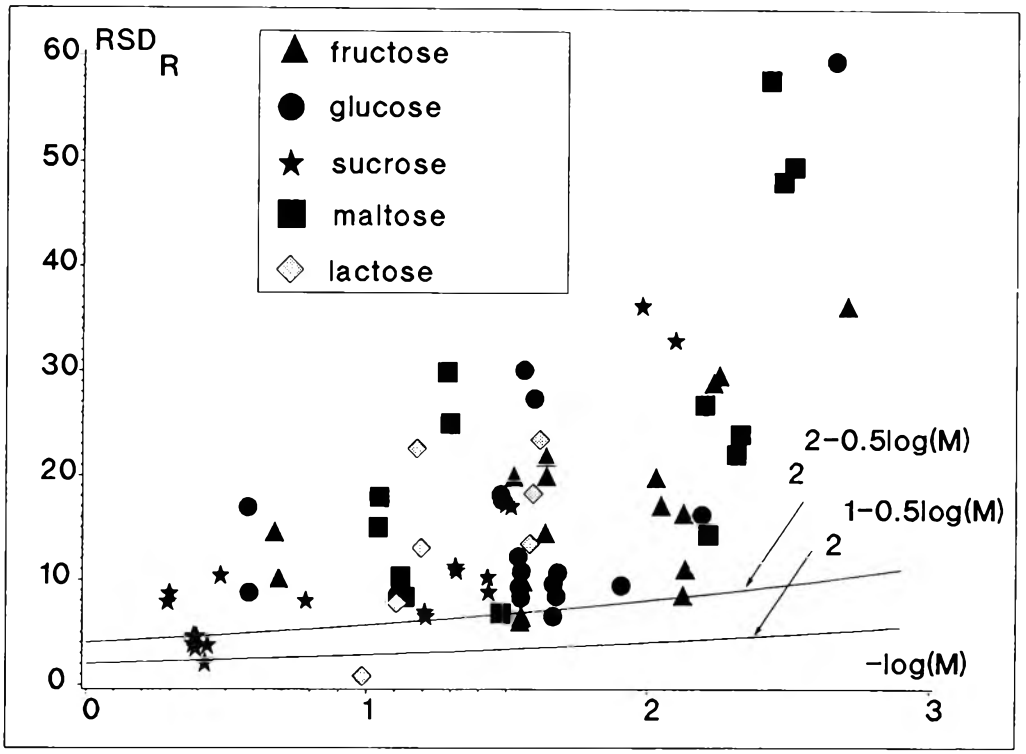

Figure 1. Representation of observed and theoretical RSD_R as a function of average concentration.

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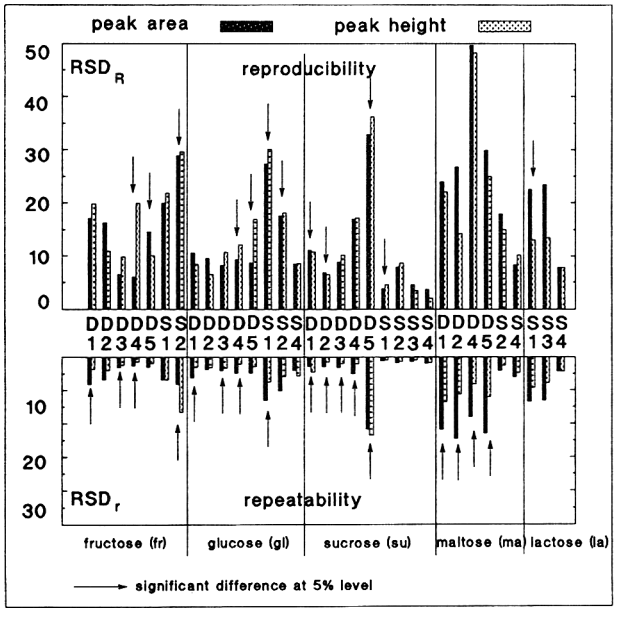

Figure 2. Comparison between peak area and peak height for precision estimation.

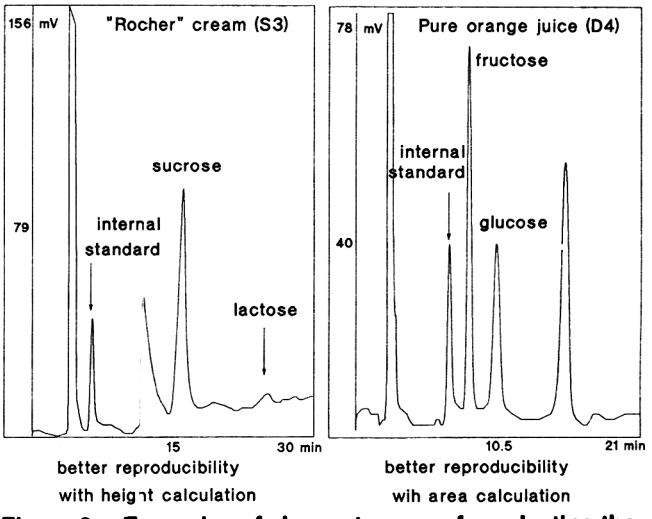

Figure 3. Examples of chromatograms for selecting the method of calculation.

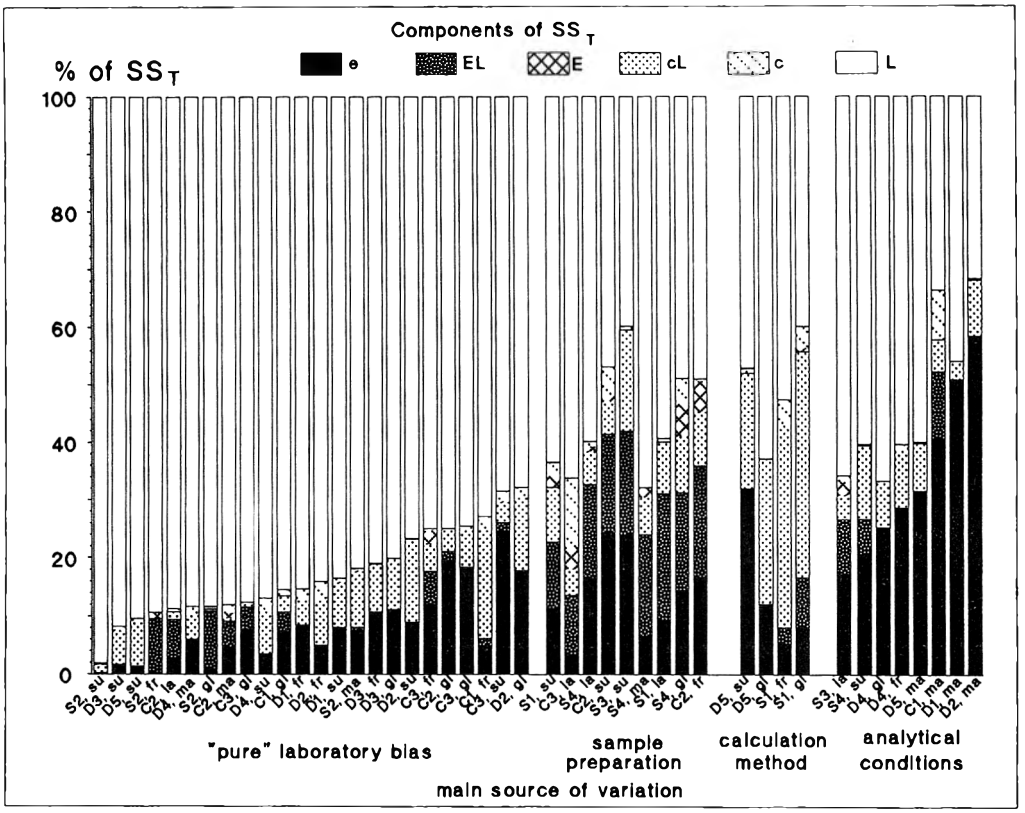

Figure 4. Decomposition of the total sum of squares SS_T: e, residual effect; EL, interaction laboratory * sample preparation; E, sample preparation effect; cL, interaction laboratory * calculation; c, calculation method effect; and L, "pure" laboratory bias.

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MICROBIOLOGICAL METHODS

Quantitation of Microorganisms in Raw Minced Meat Using the Direct Epifluorescent Filter Technique: NMKL[1] Collaborative Study

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Fourteen Nordic laboratories participated in an interlaboratory study of microorganisms in raw minced meat. After 2 preliminary collaborative evaluations of 20 and 6 prepared direct epifluorescent filter technique (DEFT) slides, the equipment and the counting technique were adjusted and standardized. In the following third and final trial, the laboratories examined 10 samples in duplicate. A special model was developed for homogenization, preservation, and transport of raw minced meat within 24 h. Test microorganisms were those present naturally in the meat. The participating laboratories received identical samples in duplicate at 10 counting levels. The results indicated a coefficient of variation of 15% by interlaboratory counting of 26 prepared DEFT slides. By examining samples of raw minced meat for microorganisms showing any degree of orange fluorescence, the repeatability and the reproducibility were 0.41 and 0.78, respectively. The repeatability standard deviation (s_r) was 0.14, and the reproducibility standard deviation (s_R) was 0.27. The study demonstrated that DEFT is a dependable method for quantitation of microorganisms in raw minced meat. Precision of DEFT was in agreement with Nordic Committee on Food Analysis standard deviation values used in the Nordic countries for plate-count quality control.

The direct epifluorescent filter technique (DEFT) is a rapid method for the quantitation of microorganisms in foods such as milk, meat, poultry and poultry products, fish and fish products, fruit and vegetables, beer and wine, and irradiated foods and water. The method was used in an interlaboratory study for examination of raw minced meat, and it provided information within 30 min.

Pettipher (1) developed the method especially for milk examination. In the Nordic countries, DEFT was tested mostly on solid foods, especially raw minced meat. The Environmental and Food Control Unit (MLK) in Odense, Denmark, tested this technique by an in-house validation study (2). MLK elaborated a draft standard for the Nordic Committee on Food Analysis (NMKL) for detection of microorganisms in raw minced meat. Pettipher et al. (3) conducted a collaborative trial, and Neaves et al. (4) conducted an interlaboratory trial using milk samples to obtain information about repeatability and reproducibility of the method and the relationship between DEFT count and plate count. In these 2 studies, 6 and 8 laboratories participated, but no determination was made on meat samples.

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This report was presented in part at the Sixth International Congress on Rapid Methods and Automation in Microbiology and Immunology, June 7–10, 1990, at Helsinki-Espoo, Finland. The method was accepted as an official NMKL-method at the 44th annual meeting of the Nordic Committee on Food Analysis, August 29, 1990, at Copenhagen, Denmark.

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The purpose of the present study was to determine the repeatability and the reproducibility of DEFT, with raw minced meat as test material, by collaborative testing with reference to an official NMKL standard (5) for quantitation of microorganisms in raw minced meat. NMKL is an independent agency that has been developing official chemical and microbiological methods that are widely used in the Nordic countries. It works in close cooperation with AOAC International. The study was designed according to International Standard ISO 5725 for precision of test methods (6).

Collaborative Study

Different types of laboratories were invited and selected through personal contacts. Fourteen Nordic laboratories participated in this interlaboratory study: 3 each from Finland, Norway, and Sweden, and 5 from Denmark. Each laboratory had an operator and a study coordinator or supervisor. The General Referee and the Nordic panel familiar with the test method planned the experiment. The Associate Referee was responsible for the following: selection of the method, organization of all facets of the study, sample preparation and distribution, conduct of the collaborative study, evaluation of results, and preparation of the final report. The study was intended for international consideration.

Preparation of Test Material

In a collaborative study, the participating laboratories must receive identical homogeneous test material. Thus, a special model for homogenization, preservation, and transportation of raw minced meat within 24 h was developed in a pilot study. Two collaborators took part in this pilot study. Test organisms used were those naturally present in the meat.

Because bacteria in raw minced meat are not uniformly dispersed, 7 samples of a total of 3 kg were further minced and mixed twice for 10 min to investigate and attain the highest obtainable level of homogeneity. All of the 10 g samples (a total of 105) were examined by visual DEFT count, semiautomatic DEFT count, and plate count. The 2 latter parameters were included only for development and evaluation of the homogenization procedure in the preparation study. The model for homogenization of raw minced meat was tested 7 times at the laboratory of the Associate Referee before the collaborative study was performed. DEFT count of the meat samples was between 10^6 and 10^8/g.

Preservation, Packaging, and Transportation

Deep freezing at –20°C was chosen for preservation. No sample was stored for more than 10 weeks, and all parallel samples were frozen in exactly the same period. Deep freezing did not affect total DEFT count, which was demonstrated when the preservation model was developed (data not shown). The purpose was to maintain the bacteria at identical levels in all samples from the homogenization stage to delivery at the participating laboratories. The freezing treatment did not affect DEFT assay, e.g., the acridine orange staining. This was demonstrated by the visual impression in the microscope field of view when 35 samples of meat were examined, first unfrozen and then after frozen storage.

The transportation model was developed after numerous experiments. The package consisted of a specially designed polystyrene container containing 6 freezing elements. Thirty meat samples packed in homogenization bags and a tube containing glycol (for determining temperature at receiving time) were put into each container and the containers were closed. A prechilled thermometer was to be used when reading the temperature in the container on receipt.

The containers were delivered by the local postal service and the air delivery service within 26 h from the time they left the laboratory of the Associate Referee. All samples were received in frozen condition. Detailed instructions of the procedure for thawing and DEFT treatment were enclosed. Advance instructions emphasized that all laboratories must start the examinations on the same day at 10 a.m.

Trial 1: Counting Prepared DEFT Slides

In Trial 1, 2 identical sets of 10 coded, prepared DEFT slides, together with a nonfluorescing microscopy oil, were circulated among the laboratories, which had been divided into 2 groups. The first group, Laboratories 1–8, consisted of 8 laboratories representing Denmark and Sweden, and the second group, Laboratories 9–14, consisted of 6 laboratories representing Norway and Finland. This part of the collaborative study was intended to familiarize the participants with the materials and methods. The slides circulated were made with different bacterial levels. The purpose was to test whether the participating laboratories were able to obtain identical counts for orange fluorescing DEFT units in the same preparation.

Trial 2: Standardization of Counting DEFT Units

The results of Trial 1 show that the laboratories did not perform the counting exactly in the same way. The visual distinction between orange, weak orange, and orange-yellow fluorescing bacteria was difficult when the laboratories were asked only to count orange fluorescing DEFT units. Therefore, a second trial including 6 coded, prepared DEFT slides was performed. The same material was circulated among all 13 participating laboratories. One laboratory (No. 10) was omitted because of time constraints. In contrast to the first trial, 2 groups of DEFT units were counted separately. One group consisted of orange fluorescing bacteria, the other of weak orange and orange-yellow fluorescing microorganisms. After that, the 2 separate DEFT counts were added, giving a total DEFT count. A nonfluorescing microscopy oil was sent, together with DEFT slides, to be used also in Trial 2. The results of the 2 preliminary collaborative evaluations of a total of 26 prepared DEFT slides were used when the equipment and counting techniques were adjusted and standardized.

Trial 3: Examination of Raw Minced Meat by DEFT

In the third and final trial, the Associate Referee sent 12 laboratories (No. 12 had no filtration equipment) the test material, consisting of 10 coded samples of frozen minced

meat (beef and pork) in triplicate (A, B, and C). Each sample was weighed (10 g) into double bags consisting of an outer stomacher bag and an inner nylon filter mesh bag. On receipt of the samples, each collaborator was instructed to examine 10 samples of different bacterial levels in duplicate (A and B) (7). The 10 pairs of samples were selected so that there would be different numbers of DEFT units in the field of view. The average levels were 34, 97, 34, 12, 20, 19, 145, 21, 8, and 27 per field. If an accident happened at any laboratory, the additional C sample was to be used (7). Blank DEFT slides for a negative control were included in the test. The laboratory of the Associate Referee had extra complete sets of test samples. Collective weighing in double bags was chosen to assure homogeneous sample volumes and to prevent drip by thawing. If the number of DEFT units per field was higher than 100, collaborators were instructed not to return to the prefiltered sample and perform a new dilution. In one sample, the counting level was selected to be higher than 100. To serve as a positive control, specially prepared DEFT slides from Trial 2 were used and a color picture with DEFT units was enclosed to serve as guidelines for the counting. Each trial of the collaborative study was performed during a 1-month period for a total of 3 months.

Direct Rapid Epifluorescent Filter Method for Assessing the Quality of Raw, Minced Meat

Principle

A known volume of sample is pretreated with an enzyme, trypsin, and a surfactant, Triton X-100. The pretreated sample is filtered through a membrane filter with an underpressure not exceeding 25 mm Hg to concentrate the microorganism on the filter. These microorganisms are stained with a fluorochrome, acridine orange (AO), and counted in a fluorescence microscope. Microorganisms cause orange and orange-yellow fluorescence under illumination with blue light at 450–490 nm because AO molecules are bound as a polymer to single chains of ribonucleic acid in actively dividing microorganisms but are bound as monomers to the double helix of deoxyribonucleic acid in inactive and dead microorganisms. The monomer shows green fluorescence. DEFT count per gram of minced meat is calculated by using the microscope factor. The total count of viable microorganisms (DEFT units) is defined as the total number of separate orange, weak orange, and orange-yellow fluorescing microorganisms together with chains and clumps of such microorganisms.

Apparatus and Glassware

(a) *Apparatus for membrane filtration of suspensions.*—Use filtration equipment of stainless steel or glass; bottom filter of sintered glass or stainless steel with a diameter of 25 mm; filter tower volume at least 10 mL.

(b) *Equipment for prefiltration of samples.*—Autoclavable filter holders, 25 mm diameter.

(c) *Equipment for sterile filtration of reagents.*—Filter funnel with suitable suction flask.

(d) *Membrane filters.*—White polycarbonate filter, 25 mm diameter, 0.4 μm pore size.

(e) *Prefilters.*—Polypropylene, 25 mm diameter, 10 μm pore size.

(f) *Filters for sterile filtration.*—Cellulose ester filters, or equivalent, 30 and 47 mm diameter, 0.2 μm pore size.

(g) *Microscope.*—Fluorescence microscope with suitable filter combination.

(h) *Optics.*—100× oil-immersion objective and 10× ocular magnification.

(i) *Slides.*—Microscope slides, 76 × 26 mm.

(j) *Slips.*—Cover slips, 50 × 25 mm thickness, corresponding to requirements of objective.

(k) *Immersion oil.*—Nonfluorescing oil, refractive index 1.515–1.518.

(l) *Stage micrometer.*—Micrometer slide for measuring diameter of the microscope field, 0.01 mm.

(m) *Homogenization equipment.*—Homogenizer of stomacher type, with sterilized plastic homogenization bags containing prefiltration bags of polyamide, for example, with a mesh size of ca 1 mm^2. The prefiltration bag should be 2 cm narrower and ca 10 cm longer than the plastic bag.

(n) *Water bath.*—Suitable for incubating samples at 50 ±1°C.

(o) *Syringes with plastic tubes.*—Disposable syringes, 10 mL, supplied with a 15 cm plastic tube.

Reagents and Diluents

(a) *Diluent.*—Dissolve 8.5 g sodium chloride and 1.0 g peptone in 1000 mL water, pH 7.2 ±0.1 at 20°C, and autoclave 15 min at 121 ±1°C.

(b) *Buffer, pH 3.0.*—100 mL 0.1M citric acid and 54 mL 0.1M sodium hydroxide, adjusted to pH 3.0 ±0.2 and then sterile-filtered.

(c) *Buffer, pH 6.6.*—35.5 mL 0.1M citric acid and 100 mL 0.1M sodium hydroxide, adjusted to pH 6.6 ±0.2.

(d) *Acridine orange (AO) solution.*—Dissolve 0.025 g AO in 100 mL buffer, pH 6.6, and filter. Concentrated AO solution is also commercially available (DEFT buffered acridine orange concentrate, Difco Laboratories, Detroit, MI 48232) and is recommended because AO is regarded as a mutagenic substance.

(e) *Enzyme.*—Rehydrate desiccated tryptic enzyme (Bacto Trypsin, Difco) with 10 mL sterile distilled water and sterile-filter [tryptic activity equivalent to 5% trypsin solution (1 + 250), Difco]. Dispense 0.5 mL portions in sterile, capped tubes and store in deep freeze if not used the same day.

(f) *Surfactant.*—Mix Triton X-100 (E. Merck, Darmstadt, Germany) with warm (80°C) distilled water in 3 concentrations: 0.1% (v/v) and 0.5% (v/v) as pretreatment reagents, and 1% (v/v) as cleaning solution; sterile-filter.

(g) *Distilled, sterile-filtered water.*

(h) *Isopropanol, 95% (v/v).*

Pretreatment for DEFT Slide Preparation

Measure 90 mL diluent into bag containing 10 g meat and homogenize 30 s. Withdraw 20 mL sample suspension from aqueous phase between plastic bag and filter bag, using pipet,

and centrifuge 30 s at 100 × g. Transfer supernatant to syringe supplied with plastic tube and further prefilter through 10 μm filter mounted in filter holder placed on syringe. Collect prefiltered sample and examine further. Treat 2 mL sample with 0.5 mL trypsin solution and 2 mL 0.5% Triton X-100 by mixing 5 s with Whirlmixer. Cap mixture immediately, and incubate 10 min in 50°C water bath. Use sterile-filtered water for control instead of sample.

Membrane Filtration

Clean polycarbonate membrane filtration equipment for sample suspensions by filtering 3 times with prewarmed, sterile-filtered 1% Triton X-100 and rinse by filtering 3 times with boiling water. Mount 0.4 μm white polycarbonate filter with shiny side up in filtration equipment. Rinse filtration tower and prewarm by filtering 5 mL warmed 0.1% Triton X-100 before filtering pretreated sample. Rinse emptied test tube with 5 mL prewarmed 0.1% Triton X-100 and filter this solution. Clean filtration columns between treatments of different samples.

Staining Membranes

Transfer 2 mL AO to filtration tower and leave on membrane 2 min. Apply vacuum and suck off staining solution. Maintain vacuum and immediately rinse membrane with 2 mL pH 3.0 buffer. Finally, rinse membrane by rapid filtration with 2 mL isopropanol. Rinsing with isopropanol must be done rapidly to avoid decolorizing microorganisms.

Mounting Membranes on Slides

Disconnect vacuum, remove membrane with pair of forceps, and dry in air. Place drop of immersion oil in middle of microscope slide. Place filter on center of oil drop with shiny side up, then place slightly smaller drop of immersion oil on membrane and put cover slip on oiled membrane. Reduce thickness of oil layer by pressing cover slip against slide. DEFT slide is now ready for examination. Slides can be stored at least 2 months at room temperature in dark.

Criteria for Counting DEFT Units

(a) *Visual counting.*—Use cross-ruled ocular or an ocular divided into squares for visual counting.

(b) *Microorganisms.*—Do not count visually orange, weak orange, and orange-yellow fluorescing particles that cannot be identified as microorganisms.

(c) *Green fluorescence.*—Do not count green fluorescing DEFT units. If visual field is fluorescing green in preparation dominated by orange and orange-yellow DEFT units, do not count green fluorescing field.

(d) *Membrane periphery.*—Do not place objective too near outside of slide. Perform count inside membrane area exposed to filtered sample.

(e) *Microscope field periphery.*—Count DEFT units appearing in periphery of microscope field only if either whole solitary microorganism or at least 1 microorganism as part of cluster or chain is visible.

(f) *Fading.*—To keep slides from fading, illuminate preparation only for period needed for examination.

Counting DEFT Units

Examine preparations in fluorescence microscope with suitable filter combination, using 100× oil-immersion objective. Place droplet of immersion oil on cover slip. Count orange, weak orange, and orange-yellow fluorescing microorganisms (DEFT units) in randomly chosen microscope fields. Number of fields counted per preparation will vary due to different numbers of DEFT units in fields. For less than 20 units, count 20 cells. If number of DEFT units in field varies from 20 to 100, count 10 fields. In this study, 1 sample containing more than 100 DEFT units per field was counted. If, in routinely used DEFT procedure, the number of DEFT units is more than 100 per field, prepare higher dilution of prefiltered sample and repeat procedure.

Count each separate microorganism or group of microorganisms separated from one another by less than twice the smallest diameter of microorganisms as 1 DEFT unit. Count clumps and chains and separate microorganisms of different morphology as different DEFT units, even for microorganisms less than 2 cell-diameters apart from each other.

Reporting Results

Calculate DEFT count (X)/g of raw minced meat by multiplying mean number of DEFT units in microscope fields examined (N/n) by dilution factor (DF) and microscope factor (MF):

$$X = (N \times MF \times DF)/n$$

where N is sum of counted DEFT units in n microscope fields; n is number of microscope fields examined; MF is microscope factor = $FA/(MF \times V)$; FA is area of membrane filter; MA is area of microscope field of view; and V is volume of sample.

Give result as DEFT count per gram of raw minced meat to 2 significant figures; round off to nearest multiple of 10.

Results and Discussion

Microscope Factor

The objective of this study was to determine the repeatability and reproducibility of DEFT under routine laboratory conditions. Laboratories were responsible for purchasing their own equipment, and this resulted in different combinations of microscopes, filter funnels, etc. However, 10 laboratories had the same brand of microscope. The microscope factor varied from 40 000 to 75 000 in Trial 1 and from 40 000 to 60 000 in the second and final trials.

Preliminary Trials

In planning the collaborative study, we decided to perform a preliminary trial in which each participating laboratory was instructed to count 10 DEFT slides prepared in the Associate Referee's laboratory. The purpose was to test the ability of the collaborators to obtain identical counts of orange fluorescing DEFT units in the same preparation. The results of Trial 1 demonstrated that the laboratories did not perform the counting technique in exactly the same way. The visual distinction between orange, weak orange, and orange-yellow fluorescing

DEFT units was especially difficult when the laboratories were asked only to count orange fluorescing DEFT units as viable bacteria. Green fluorescing DEFT units should not be counted, but in some fields of view, weak orange and orange-yellow fluorescing bacteria could occur together with orange fluorescing bacteria.

For both of the 2 identically prepared sets in Trial 1, each with 10 DEFT slides, a statistical analysis of variation was made from data of DEFT counts to test the difference between average DEFT counts of the 2 laboratory groups. At a probability level of 95%, an average DEFT count without significant difference was found for 7 of the sets of DEFT slides. For 3 sets, the average DEFT count was significantly lower for laboratory group 9–14 than for laboratory group 1–8 (data not shown).

On the basis of results of Trial 1, a second trial was conducted in which all collaborators counted the same 6 prepared DEFT slides. In Trial 2, the participants were instructed to count orange fluorescing DEFT units separately from weak orange and orange-yellow DEFT units. In addition, the instruction for counting DEFT units was further defined. More uniform results were obtained by the collaborators when the 2 separate counts were added together.

Stability Test of DEFT Slides

To evaluate the stability of the prepared DEFT slides, the set circulated among Laboratories 1–8 was counted visually in the Associate Referee's laboratory both before and after it was circulated among the collaborators. This investigation showed that the prepared slides did not change during circulation.

Homogenization Model

In Trial 3, the samples of minced meat were homogenized to disperse the microorganisms uniformly. The samples were minced and mixed twice and were examined 5 times after each preparation. By following this procedure and repeating it many times, we found that the difference between maximum and minimum DEFT count could not be improved beyond log 0.22 when the same sample was examined 5 times in the Associate Referee's laboratory, using repeatability conditions of ISO 5725 (6). The result can be estimated as good for naturally contaminated minced meat samples (data not presented).

Pretreatment

In this study, several pretreatment steps were used to prepare DEFT slides with high fluorescence signal from microorganisms and minimum noise from fluorescing meat particles. The purpose of the pretreatment was to transfer all microorganisms of the meat sample to an aqueous solution suitable for membrane filtration. After the homogenization, 20 mL sample suspension was withdrawn with a pipet, but the elimination of clogging problems from food debris was not possible. To solve these problems, the Associate Referee devised a new double bag method (2) consisting of an outer stomacher plastic bag and an inner filter bag made of fine-meshed polyamide. This double bag was used in the collaborative study to improve the pretreatment. Before the meat sample was weighed, the sterile prefiltration polyamide bag was placed inside the stomacher plastic bag. The subsequent 30 s homogenization achieved the effect of a "tea bag," retaining the solid meat components in the filter bag and at the same time washing out the microorganisms in the aqueous dilution phase between the filter bag and the plastic bag. The withdraw of the desired 20 mL sample suspension from the aqueous phase into a test tube was simplified. The test tube was centrifuged briefly so that the coarsest meat particles were deposited. (This centrifugation is very helpful for the further membrane prefiltration through the 10 μm filter.) Finally, surfactant and enzyme were used, followed by polycarbonate membrane filtration, AO-staining, and rinsing, resulting in DEFT slides of good quality, i.e., without any or with only a few autofluorescing particles.

Used as described above, the combined pretreatment steps are an improvement of DEFT for examining raw minced meat, and they give better repeatability and reproducibility. DEFT slides of good quality are a prerequisite for the counting technique, both visual and semiautomated. In this study, both counting techniques were used in the Associate Referee's laboratory when the homogenization model was developed, whereas the participating laboratories used only the visual DEFT count in Trial 3. All collaborators were asked to perform semiautomatic DEFT counts if they had the required equipment, but only 5 laboratories had an image analyzer system. Therefore, semiautomatic DEFT counts were excluded from this study. Results demonstrated, however, that the semiautomated counting technique is suitable for DEFT slides used in the study, which were made by the combined pretreatment procedure (data not presented).

Final Trial

Results from all participating laboratories were reported to the Associate Referee on a standard report form giving visually counted DEFT units, microscope factor, DEFT count, and details of collaborators and any deviation from the prescribed method.

To compare Trials 1, 2, and 3, the coefficient of variation was computed; it averaged 15% in Trials 1 and 2 and 38% in the final trial. This sort of increase in variation is common when all sources of within-laboratory and between-laboratories variation are considered.

In Trial 3, the difference between maximum and minimum DEFT counts was calculated for every minced meat sample. By counting the orange fluorescing DEFT units separately from the group of orange, weak orange, and orange-yellow fluorescing DEFT units, the overall average difference between maximum and minimum DEFT counts in A-samples decreased from 1.46 to 0.93. When the matched B-samples were examined, the decrease was from 1.01 to 0.86. This indicates greater assurance in counting the total amount of orange, weak orange, and orange-yellow microorganisms than in counting only orange microorganisms.

Figure 1 presents results for the determination of DEFT count ($\log_{10}$ count/g) in matched pairs of meat Samples 1–5 A and 1–5 B. Similarly, results for the determination of DEFT count ($\log_{10}$ count/g) in matched pairs of meat Samples 6–10 A and 6–10 B are shown in Figure 2. The horizontal line in each

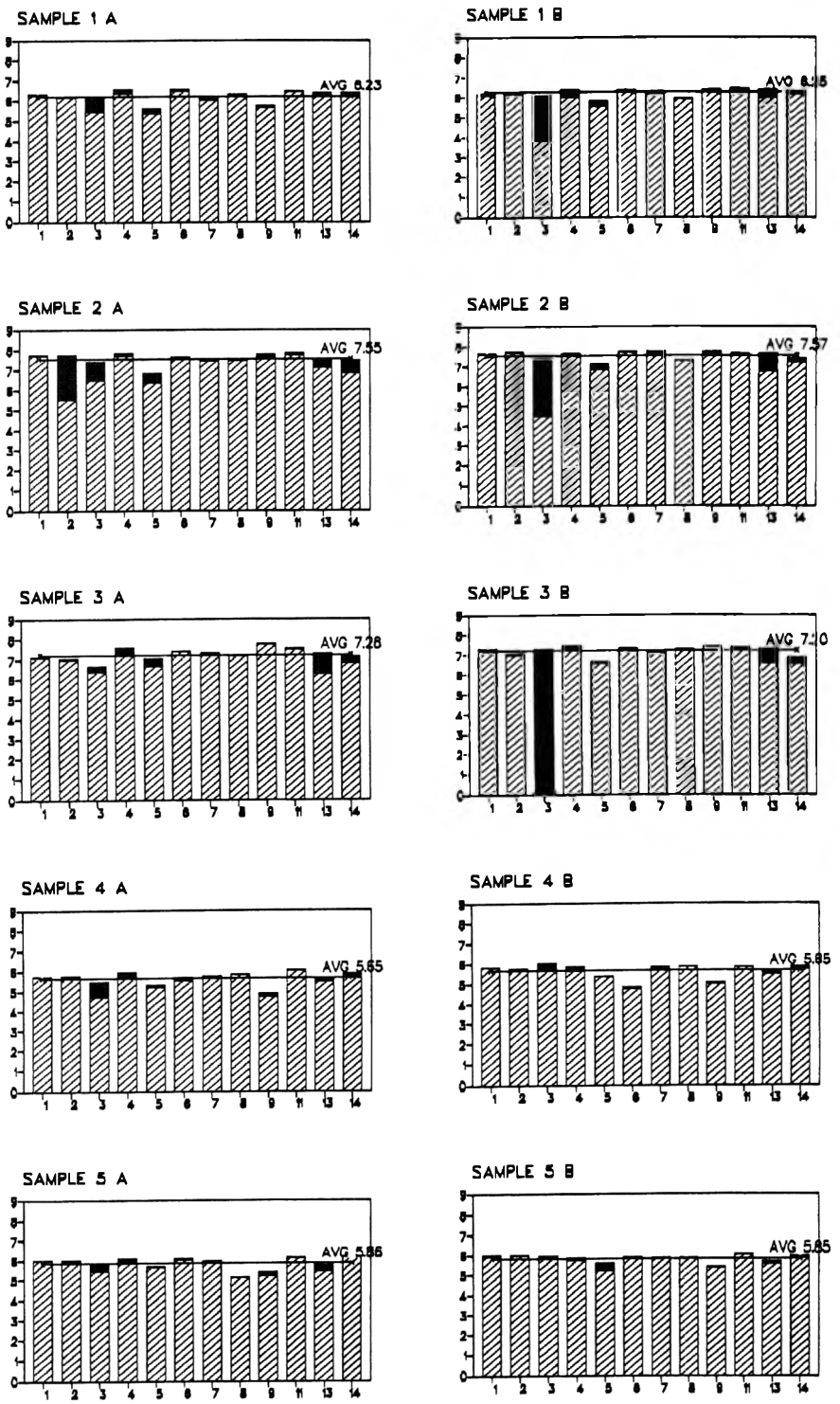

Figure 1. Determination of DEFT count (log_{10} count/g) in 5 matched pairs (Sample 1–5 A and Sample 1–5 B) of raw minced meat: x = Laboratory, y = log_{10} DEFT count/g, ▨ = orange DEFT units, ■ = weak orange and orange-yellow DEFT units.

figure represents the average DEFT count for all participating laboratories. Laboratories 10 and 12 did not participate in the final trial. DEFT counts were converted to log_{10} count/g before statistical analysis. We assumed that the log_{10} counts would be normally distributed and of homogeneous variance.

For the determination of the precision of DEFT in this study, a statistical analysis (6) was applied using Cochran's outlier test in which repeatability, reproducibility, repeatability standard deviation, and reproducibility standard deviation of the final trial were determined. Because the exact values of the

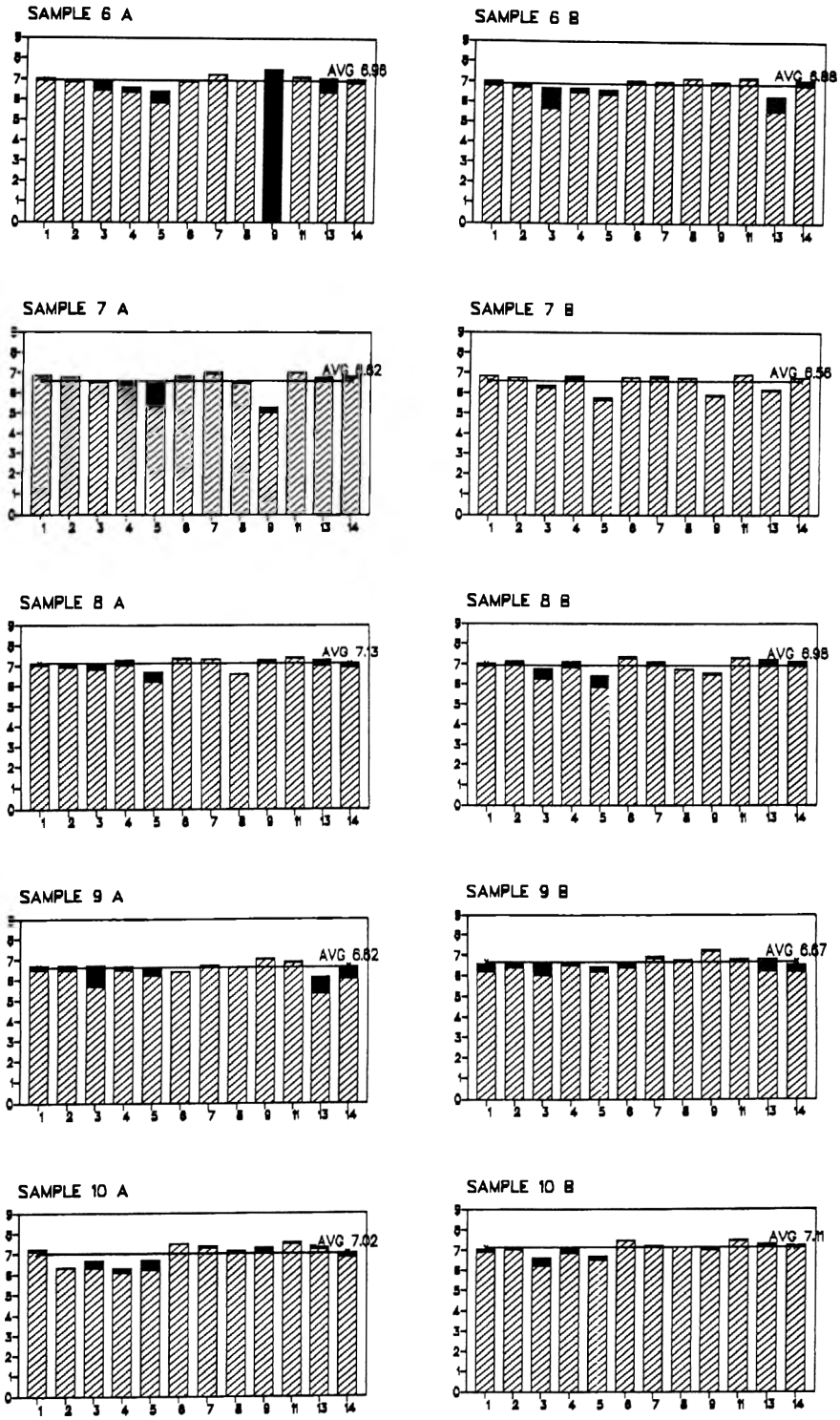

Figure 2. Determination of DEFT count (log_{10} count/g) in 5 matched pairs (Sample 6–10 A and Sample 6–10 B) of raw minced meat: x = Laboratory, y = log_{10} DEFT count/g, ▨ = orange DEFT units, ■ = weak orange and orange-yellow DEFT units.

repeatability standard deviation and the reproducibility standard deviation are not known in practice, they are replaced by their estimates (s), leading to the following formulas used in the study:

$$r = 2.83 \times s_r, \text{ and } R = 2.83 \times s_R.$$

These data of the statistical analysis of DEFT counts (log_{10} count/g) are reported in Tables 1 and 2. Not more than 1 result in 12 (8.3%) was deleted from each parallel sample as a result of the outlier test. This is well below the IUPAC recommended maximum of 22.2% (8). When only orange fluorescing bacte-

Table 1. Statistical analysis of data from DEFT counts (log_{10} count/g) in 10 matched pairs (A and B) of raw minced meat samples when only orange fluorescing DEFT units were counted

Parameter	Sample No. 1	2	3	4	5	6	7	8	9	10	1–10
No. of laboratories after eliminating outliers	11	12	11	12	12	11	11	12	11	12	
No. of outlying laboratories removed	1	0	1	0	0	1	1	0	0	0	
Mean (log_{10} DEFT count/g)	6.12	7.17	7.08	5.53	5.76	6.67	6.57	6.89	6.46	6.94	
Repeatability SD (s_r)	0.18	0.63	0.13	0.27	0.22	0.26	0.16	0.21	0.12	0.25	
Repeatability rel.SD (RSD_r)	2.94	8.78	1.83	4.88	3.81	3.89	2.43	3.04	1.85	3.60	
Repeatability value, r (2.83 × s_r)	0.52	1.79	0.39	0.78	0.64	0.75	0.46	0.60	0.34	0.71	
Reproducibility SD (s_R)	0.27	0.80	0.36	0.38	0.31	0.46	0.41	0.37	0.33	0.44	
Reproducibility rel.SD (RSD_R)	4.41	11.15	5.08	4.88	5.38	6.89	6.24	5.37	5.10	6.34	
Reproducibility value, R (2.83 × s_R)	0.79	2.28	1.04	1.08	0.88	1.33	1.17	1.07	0.96	1.26	
Overall repeatability SD (s_r)											0.24
Overall repeatability value (r)											0.69
Overall reproducibility SD (s_R)											0.41
Overall reproducibility value (R)											1.18

ria were counted, the overall average repeatability value (r) was 0.69, and the overall average reproducibility value (R) was 1.18. The corresponding values when orange, weak orange, and orange-yellow microorganisms were counted were 0.41 and 0.78, respectively. The precision for DEFT applied to raw minced meat was better in the latter examination, which did not require differentiation between shades of orange. At the 95% confidence interval, the standard deviation was 0.14 for the overall average repeatability (s_r) and 0.27 for the overall average reproducibility (s_R).

In a NMKL report (9), s_r values of 0.10–0.15 and s_R values of 0.20–0.25 were given for plate count quality control. In our study, DEFT showed precision in agreement with these values. The method is entirely adequate for use in a quality control system for raw minced meat.

Recommendation

On the basis of the results of this collaborative study, DEFT has demonstrated its practical value for the quantitation of microorganisms in raw minced meat. DEFT can be used in public food control as well as in the food industry for control of production quality and quality assurance of raw material. Also, it can be used as a convenient monitoring tool for semimanufactured products when applying the Hazard Analysis Critical Control Point concept. The result is obtained within 30 min.

Table 2. Statistical analysis of data from DEFT counts (log_{10} count/g) in 10 matched pairs (A and B) of raw minced meat samples when orange, weak orange, and orange-yellow fluorescing DEFT units were counted

Parameter	Sample No. 1	2	3	4	5	6	7	8	9	10	1–10
No. of laboratories after eliminating outliers	11	12	12	11	11	11	12	11	11	12	
No. of outlying laboratories removed	1	0	0	1	1	1	0	1	1	0	
Mean (log_{10} DEFT count/g)	6.25	7.56	7.23	5.68	5.88	6.94	6.58	7.06	6.66	7.06	
Repeatability SD (s_r)	0.10	0.09	0.18	0.14	0.09	0.14	0.22	0.11	0.10	0.23	
Repeatability rel.SD (RSD_r)	1.60	1.19	2.48	2.46	1.53	2.01	3.34	1.55	1.50	3.25	
Repeatability value, r (2.83 × s_r)	0.30	0.27	0.53	0.42	0.26	0.40	0.64	0.33	0.29	0.66	
Reproducibility SD (s_R)	0.22	0.24	0.26	0.31	0.21	0.24	0.40	0.27	0.20	0.36	
Reproducibility rel.SD (RSD_R)	3.52	3.17	3.59	5.45	3.57	3.45	6.07	3.82	3.00	5.09	
Reproducibility value, R (2.83 × s_R)	0.63	0.68	0.75	0.90	0.62	0.69	1.16	0.79	0.58	1.02	
Overall repeatability SD (s_r)											0.14
Overall repeatability value (r)											0.41
Overall reproducibility SD (s_R)											0.27
Overall reproducibility value (R)											0.78

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MYCOTOXINS

Bioassay, Extraction, and Purification Procedures for Wortmannin, the Hemorrhagic Factor Produced by *Fusarium oxysporum* N17B Grown on Rice

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Rats and cultured cells were treated with purified wortmannin toxin, crude fungal extracts, and partially purified preparations for periods of 5–96 h. A single dose of the toxic fractions containing the equivalent of 2.5 g fungus-infested rice resulted in death, hematuria, and hemorrhage in the urinary bladder, intestine, stomach, heart, and thymus of rats. Purified wortmannin produced the same effects at 4 mg/kg. Fractions that were toxic in vivo also had cytotoxic properties with various cultured cell lines at concentrations ranging from 1.2 to >50 mg equivalents of fungus-infested rice extracted/mL. Purified wortmannin was cytotoxic at 50 μg/mL.

Fusarium oxysporum Schlecht. emend. Snyder & Hansen is commonly found on various commodities and in soil samples (1–3). Of the compounds responsible for toxicity in animals, the only ones that have been evaluated are diacetoxyscirpenol (DAS), T-2 toxin, zearalenone (ZEA) (4–7), moniliformin, and fusaric acid (8). Ghosal et al. (9) reported that isolates of *F. oxysporum* caused skin necrosis in rats; among the toxins produced were trichothecenes, 2 of which were identified as DAS and T-2 toxin. One of 38 isolates of *F. oxysporum* from *Baccharis* spp. originating in Brazil produced congestion and hemorrhage of tissues and death when fed to rats (10). Yang et al. (11) reported a correlation between Kashin-Beck disease and the presence of *F. oxysporum* in certain grains in endemic areas of China.

Various physical assays (2, 12–14) and bioassays using animal species (2, 15–21) or cultured cell types (2, 14, 22, 23) have been developed to study mycotoxins from *Fusarium* spp. Because food materials infested with species of *Fusarium* may contain substances that produce animal and human toxicities (2, 24–27), we have developed procedures for the bioassay, extraction, and purification of the major hemorrhagic factor produced by an isolate of *F. oxysporum* (N17B).

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Experimental

Fungus Culture

Species were identified in the laboratory of T. Kommedahl, Department of Plant Pathology, University of Minnesota, by the method of Nelson et al. (28). Stock cultures of this isolate were maintained in moist, autoclaved soil stored at −15°C. *F. oxysporum* N17B was grown on rice medium as described by Abbas and Mirocha (2).

Preparation of Extracts of F. oxysporum N17B-Infested Rice

Crude extracts of *F. oxysporum* N17B-infested rice medium were prepared with 10 different solvents: methylene dichloride, chloroform, acetonitrile, acetone, ethyl acetate, 50% ethyl acetate in acetonitrile, methanol, water, 50% methanol in water, and 50% acetonitrile in water. Ground, dried 50 g samples of medium moistened with 25 mL water were extracted with one of the test solvents (3 times for 1 h at 24°C; total of 300 mL). The combined extracts were prepared for toxicity testing by filtering through Whatman No. 4 filter paper, evaporating to dryness in a rotary evaporator at 35°C, and dissolving the residues in 10 mL 40% aqueous (v/v) ethanol. The 50 samples of extracted fungus-infested rice medium were prepared for feeding studies by air-drying for 3 days in a ventilated hood, grinding, and mixing with complete rat diet.

Purification of Wortmannin from F. oxysporum N17B-Infested Rice

The scheme for the extraction and purification of wortmannin is shown in Figure 1. A 50 g sample of air-dried, fungus-infested rice medium was ground to the consistency of flour and moistened with 25 mL distilled water. Wortmannin was extracted from the sample by shaking 3 times with ethyl

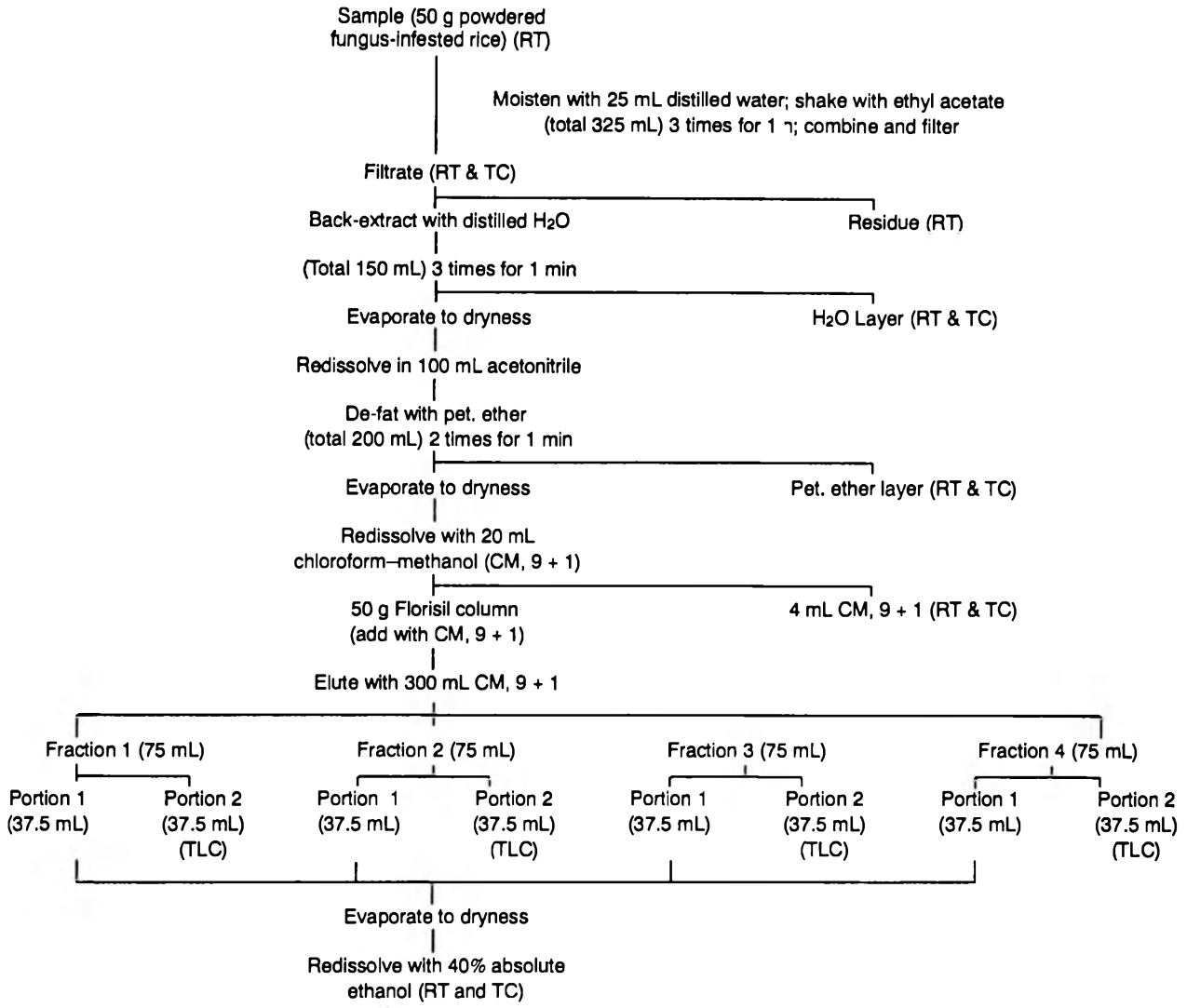

Figure 1. Bioanalytical method for hemorrhagic factor(s) in *Fusarium*-infested rice. RT = rat intubation test; TC = tissue culture cytotoxicity test; CM = chloroform–methanol; and TLC = thin-layer chromatography.

acetate for 1 h (total volume, 300 mL) in a 500 mL flask, using a wrist-action shaker at room temperature (24°C). The combined extracts were filtered through Whatman No. 4 filter paper and back-extracted with distilled water 3 times for 1 min (total volume, 150 mL) in a 1000 mL separatory funnel. The water layers were combined, evaporated to dryness under reduced pressure on a rotary evaporator at 35°C, and saved for bioassays. The ethyl acetate layer, which contained the crude wortmannin, was also evaporated to dryness under reduced pressure on a rotary evaporator at 35°C.

The residue was redissolved in 20 mL chloroform–methanol (9 + 1, v/v), and a 4 mL aliquot (equivalent to 10 g fungus-infested rice) was retained for bioassays. The remainder was applied to a 2.5 × 100 cm column containing 50 g Florisil packed in the same solvent and topped with a guard layer of Na_2SO_4 (29). The material was eluted with 300 mL chloroform–methanol (9 + 1) collected as four 75 mL fractions in 300 mL round-bottomed flasks. The fractions were evaporated to near dryness on a rotary evaporator at 35°C, and each fraction was subjected to toxicity testing and physical studies.

Wortmannin was present in the second (yellow) fraction, which was applied to 20 × 20 cm thin-layer chromatography (TLC) plates coated with a layer of silica gel 60, 0.25 mm thick, without fluorescent indicator (E. Merck, Darmstadt, Germany) and developed with chloroform–methanol (97 + 3, v/v). Bands were detected in guide strips broken from the edges of the plates, sprayed with 20% (v/v) aqueous sulfuric acid or freshly prepared acidic *p*-anisaldehyde (30), and heated to 60–70°C until the bands appeared. The TLC sheet was divided into 10 horizontal bands of adsorbent, 0.5–0.7 cm wide, which were individually scraped from the glass sheet, packed into columns, and eluted with absolute alcohol. The solvent was removed from each eluate under vacuum, and solutions for bioassay were prepared by dissolving the residues in 40% aqueous eth-

anol at concentrations equivalent to 5 g *Fusarium*-infested rice medium/mL extract. Wortmannin was present as a readily visible yellow component in Band 9.

Crystallization of Wortmannin

Enough wortmannin for crystallization and analysis was obtained by performing the procedure described above on 20 individual 50 g samples of dried *F. oxysporum* N17B-infested rice medium. The yellow bands from TLC plates were eluted together, and the eluates were evaporated to dryness and dissolved in a minimum of methanol. Crystals of wortmannin formed on standing at 20°C. Additional crystals formed on standing at 4°C for 5–6 h, then overnight at –15°C. The crystals were collected by filtration and recrystallized 3 times from methanol to yield 52 mg of white fluffy crystals per kg of dried rice medium extracted, m.p. 222–223°C. Other physical and spectral properties were described previously (2).

Oral Toxicity Testing in Rats

Rat oral toxicity tests were performed as described by Abbas and Mirocha (2) on groups of 8 individually housed Sprague-Dawley rats (20-day-old virgin females, 38–46 g, Bio-Labs, St. Paul, MN). In feeding studies, rats were fed *Fusarium*-infested rice medium mixed with complete rat diet (1:3, 1:1, and 3:1). Controls received ground noninfected rice medium mixed with complete rat diet (1:1). Extracts or fractions were administered by gastric intubation in 0.5 mL 40% aqueous ethanol containing 2.5 g equivalents of fungus-infested rice per rat per day. Control rats were administered solvent in the same manner. Treated rats were observed frequently for 24 h, and major toxic symptoms (hematuria, congestion, and hemorrhage in intestines, heart, and stomach; spleen and thymus size; diarrhea; weight loss) and death were recorded. The rats were necropsied at death and examined for pathological changes in tissues.

Cytotoxicity Determinations in Cultured Mammalian Cells

Materials were obtained as follows: 3T3 Swiss mouse fibroblasts from R.H. Holley, Salk Institute, La Jolla, CA; GM498 human diploid fibroblast line from Institute for Medical Research, Camden, NJ; and HeLa S3 human cervical epithelioid carcinoma cells from American Type Culture Collection, Rockville, MD. Unless otherwise indicated, all components of the medium were obtained from Sigma Chemical Co., St Louis, MO. Cell lines were cultured in 10% (v/v) calf serum (Hyclone Laboratories, Ogden, UT) in Dulbecco's modified Eagle's medium, supplemented with 500 units penicillin/mL and 100 μg streptomycin/mL, in a 37°C incubator with humidified atmosphere containing 12% CO_2. Cell lines were maintained by subculturing, using detachment by 0.05% (w/v) trypsin in medium, or by storage in liquid nitrogen in medium containing 10% (v/v) dimethylsulfoxide and 20% (v/v) calf serum. Primary neonatal rat beating heart cell cultures were established from litters of approximately ten 2-day-old Sprague-Dawley rat pups (Bio Labs) by the method of Blondel et al. (31), using trypsin dispersion of the tissue and a 3 h preplating period to remove mesenchyme cells. The cells were cultured as described above for 2 days before use, except for Eagle's minimum essential medium in a 5% (v/v) CO_2 atmosphere.

Cytotoxicity assays are performed essentially as described previously (2, 14). Samples in 40% aqueous ethanol were diluted into sterile medium so that the final concentration of ethanol in the culture medium was always <1% (v/v). Serial dilutions were made in triplicate in the wells of sterile 96-well culture trays (Falcon Products No. 3072, Oxnard, CA) with sterile medium as diluent. Triplicate control wells received similar dilutions of the solvent. For cytotoxicity determinations with permanent cell lines, each culture well received 100 μL medium containing the diluted sample and then an additional 100 μL medium containing 20% (v/v) calf serum and a suspension of the test cells removed from stock culture dishes with the aid of trypsin and counted in a hemocytometer. For determining toxicity with primary cultures of neonatal rat beating heart cells, cultures were established in 96-well culture trays, incubated 2 days, washed 3 times with sterile medium, and cultured with 200 μL aliquots of diluted test samples in sterile Eagle's minimal essential medium containing 10% (v/v) calf serum.

Cells were cultured in the trays for 3–4 days or until control cultures reached confluency. The cells were washed on the well bottoms with serum-free medium and fixed in ca 200 μL formal saline [10% (v/v) formalin (37%, w/v, aqueous formaldehyde), NaCl (5 g/L), and Na_2SO_4 (15 g/L)] for at least 20 min. The fixative was decanted, and the cells were rinsed with water and stained 30 min with ca 200 μL freshly diluted Giemsa stain [prepared as a 1 + 20 dilution of 8 mg/mL in methanol–glycerol (1 + 1)]. The wells were washed with water to remove excess stain. The approximate LC_{50} (the approximate concentration that results in one-half the number of cells surviving in control wells) was estimated visually. An additional evaluation of toxicity was possible with rat beating heart cells by examining cultures under an inverted-phase microscope for inhibition of spontaneous beating activity after 24 h of incubation with toxic preparations.

Results

Toxicity of F. oxysporum N17B to Rats Fed Different Ratios of Infested Rice

As an approach to developing a model of foodborne mycotoxicosis by *F. oxysporum*, 4 groups of rats were fed either a control diet consisting of a 1:1 mixture of autoclaved noninfected rice and complete rat diet or various mixtures of complete rat diet and rice inoculated with *F. oxysporum* N17B. No deaths occurred among the rats in the control group, whereas the weight gains of rats fed fungus-infested rice in their diet were substantially decreased, and the majority died by the 4th or 5th day of feeding (Table 1). Presumably, part of the reduced weight gain reflects food refusal-inducing activity in the fungus-infested rice. Rats consuming fungus-infested rice showed definite toxic effects, including hematuria in both surviving

Table 1. Toxicity of *Fusarium oxysporum* N17B-infested rice medium fed to rats at 3 concentrations

Preparations and ratios fed[a]	Av. wt. change[b]	Av. wt. food consumed, g[b]	Mortality, %	Toxicity[c]
Uninfected rice:diet, 1:1	19.7 ± 3.3	40.0 ± 2.5	0.0	—
Fungus-infested rice:diet, 1:3	−12.6 ± 1.3	7.0 ± 1.6	62.5	+, 6 H, 2 In, 1 S
Fungus-infested rice:diet, 1:1	−13.2 ± 2.5	4.1 ± 0.3	85.9	+, 4 H, 1 In, 1 S
Fungus-infested rice:diet, 3:1	−16.5 ± 0.6	3.3 ± 0.7	100.00	+, 6 H, 1 In, 2 S

[a] Uninfected rice = control autoclaved, dried, and ground uninfected rice; diet = complete rat diet; fungus-infested rice = autoclaved rice on which *F. oxysporum* N17B was grown, then dried and ground.
[b] Each value is the mean ± standard error of the mean for 8 rats.
[c] Abbreviations: − = no toxic effect; + = definite toxic effect; H = hematuria; In = intestinal hemorrhage; S = stomach hemorrhage; and numbers = number of rats showing the indicated symptoms.

and dead rats as the major clinical symptom, with minimal hemorrhage in stomach and intestines of some rats.

Selection of Solvent to Extract Hemorrhagic Factor Wortmannin Produced by F. oxysporum

Because rat feeding studies consistently demonstrated that the isolate of *F. oxysporum* N17B was the most hemorrhagenic of isolates examined, it was studied further. Ten different solvent systems listed in Table 2 were examined for efficiency in extracting hemorrhagic activity from samples of ground rice medium that had supported the growth of *F. oxysporum* N17B. Extracts prepared with methylene dichloride, chloroform, acetonitrile, acetone, ethyl acetate, and 50% ethyl acetate in acetonitrile administered daily by stomach intubation were highly toxic, as indicated by the ability of the samples to cause (*i*) death within 24–48 h, (*ii*) congestion and hemorrhage of stomach, intestine, thymus, and heart, and (*iii*) hematuria (Figure 1). The controls and extracts prepared with methanol, water, 50% methanol in water, and 50% acetonitrile in water showed no toxic effects (Table 2). Similar feeding studies on 1:1 mixtures of normal diet with extraction residues of fungus-infested rice samples indicated residual unextracted toxicity with all solvents except methylene dichloride, 50% acetonitrile in water, and ethyl acetate (Table 3). All test rats developed diarrhea and small spleens and thymuses. Because ethyl acetate was the least toxic of the solvents that left minimal toxic residue, it was selected for routine use in the extraction of the hemorrhagic factor (wortmannin).

Purification of Wortmannin

The ethyl acetate extract (Crude Extract 1) prepared from dried rice medium infested with *F. oxysporum* was highly toxic to rats as indicated by its ability to (*i*) cause death within less than 24 h in large doses and (*ii*) cause hemorrhage in bladder, stomach, intestine, thymus, and heart, and cause hematuria at lower doses (Table 4). Also, this crude extract induced cytotoxicity at low concentrations (LC_{50} values of 1.5 mg equivalents of fungus-infested rice per milliliter in 3T3, 15 mg equivalents/mL in GM498, 1.5 mg equivalents/mL in HeLa cells, and 7.5 mg equivalents/mL in beating heart cells) (Table 4).

Table 2. Toxicity of extracts of *Fusarium oxysporum* N17B cultured on rice in rats after direct intubation to the stomach

	No. rats dead					Congestion and hemorrhage[a]			
Extracts fed to groups of 3 rats	24 h	48 h	72 h	96 h	120 h	Stomach	Bladder	Heart	Intestine
40% ethanol (Control 1)	0	0	0	0	0	−	−	−	−
Complete diet extracted with ethyl acetate (Control 2)	0	0	0	0	0	−	−	−	−
Autoclaved rice-complete diet (1:1) extracted with ethyl acetate (Control 3)	0	0	0	0	0	−	−	−	−
Methylene chloride	3	—	—	—	—	+	+	+	−
Chloroform	0	3	—	—	—	−	+	+	+
Ethyl acetate	3	—	—	—	—	+	+	+	−
Acetone	2	0	1	—	—	+	+	+	−
Acetonitrile	3	—	—	—	—	+	+	+	+
Methanol	0	0	0	0	0	−	−	−	−
Water	0	0	0	0	0	−	−	−	−
50% ethyl acetate in acetonitrile	3	—	—	—	—	+	+	+	+
50% acetonitrile in water	0	0	0	0	0	−	−	−	−
50% methanol in water	0	0	0	0	0	−	−	−	−

[a] − = no detectable toxic effect; + = definite hemorrhage. All rats (average wt = 43.1 ± 0.7 g) received a daily dose equivalent to the extract of 2.5 g fungus-infested rice or the equivalent amount of control extract.

Table 3. Toxicity of residues from extraction of *Fusarium oxysporum* N17B cultured on rice medium with various solvents in groups of 3 rats fed 1:1 mixture of residue and complete rat diet

Residues from extraction with indicated solvent	Weight gain, g	Food consumption, g	Congestion and hemorrhage of tissues or death[a]		
			Stomach	Bladder	Death
Unextracted complete diet (Control 1)	29.7 ± 0.7	44.0 ± 2.1	–	–	–
Complete diet extracted with ethyl acetate (Control 2)	15.3 ± 1.4	33.0 ± 3.2	–	–	–
Autoclaved rice-complete diet (1:1) extracted with ethyl acetate (Control 3)	13.7 ± 1.3	25.7 ± 2.4	–	–	–
Methylene chloride	1.34 ± 0.3	21.7 ± 1.3	–	–	–
Chloroform	2.0 ± 1.2	18.0 ± 5.1	+	–	–
Ethyl acetate	-3.0 ± 1.0	13.0 ± 1.5	–	–	–
Acetone	-5.0 ± 0.6	21.3 ± 3.5	+	–	–
Acetonitrile	-6.0 ± 0.6	13.0 ± 4.6	+	–	–
Methanol	-7.3 ± 1.2	20.0 ± 5.1	+	+	+
50% ethyl acetate in acetonitrile	-2.0 ± 1.2	14.0 ± 1.5	+	–	–
50% acetonitrile in water	2.7 ± 1.7	17.7 ± 3.3	–	–	–
50% methanol in water	-4.3 ± 2.3	22.0 ± 2.6	+	+	+

[a] – = no detectable toxic effect; + = definite hemorrhage or death. All rats, except controls, had small thymuses, small spleens, and diarrhea.

Crude Extract 2 was prepared by back-extracting Crude Extract 1 with distilled water, evaporating the ethyl acetate layer, and defatting an acetonitrile solution of the residue with petroleum ether. It was highly toxic to rats, as indicated by the ability to cause death within less than 24 h after administration by gastric intubation and to cause hematuria and hemorrhage in bladder, intestine, stomach, and heart (Table 4). Crude Extract 2 retained most of the cytotoxicity (LC_{50} values of 15 mg equivalents/mL in 3T3, 35 mg equivalents/mL in GM498, 15 mg equivalents/mL in HeLa cells, and 1.2 mg equivalents/mL in beating heart cells) (Table 4).

Crude Extract 2 was fractionated on a Florisil column to yield 4 fractions. Florisil column Fraction 2, which was yellow, caused death in rats in less than 24 h and hemorrhage in bladder, intestine, stomach, and heart. However, it was generally less cytotoxic than the 2 crude culture extracts (LC_{50} values of

Table 4. Toxicity to rats and cultured cells of crude extracts of *Fusarium oxysporum* N17B grown on rice medium, solvent partition fractions, and florisil-column fractions

Extracts or fractions	Oral toxicity in rats[a]	Cytotoxicity (LC_{50} in mg equiv. of fungus-infested rice extracted/mL)[b]			
		3T3 mouse fibroblasts	GM 498 human fibroblasts	HeLa cells	Rat beating heart cells
40% aqueous ethanol (Control 1)	–	–	–	–	–
Ethyl acetate extract of rat diet (Control 2)	–	–	–	–	–
Ethyl acetate extract of autoclaved rice-rat diet, 1:1 (Control 3)	–	–	–	–	–
Crude Extract 1	+, 3 D, H, HB, HI, HS, HH, HT	1.5	15	1.5	7.5
Water fraction	–	–	–	–	–
Petroleum ether fraction	–	–	–	–	–
Crude Extract 2	+, 1 D, H, HB, HI, HS, HH	15	35	15	1.2
Florisil Fraction 1	–	–	–	–	–
Florisil Fraction 2 (yellow)	+, 2 D, HB, HI, HS, HH	20	50	20	50
Florisil Fraction 3	–	–	–	–	–
Florisil Fraction 4	–	–	–	–	–

[a] Toxicity of extracts equivalent to 2.5 g fungus-infested rice dissolved in 0.5 mL 40% ethanol fed daily for 5 days to groups of 3 rats by gastric intubation. Abbreviations: + = definite toxic effect; – = no detectable toxic effect; D = death; number = number of dead rats; H = hematuria; HB = hemorrhage in bladder; HI = intestinal hemorrhage; HS = hemorrhage in stomach; HH = hemorrhage in heart; and HT = hemorrhage in thymus.

[b] – = cytotoxicity at > 50 mg equiv. of fungus-infested rice extracted/mL.

Table 5. Toxicity to rats and cultured cells of TLC fractions from Florisil column Fraction 2 of *Fusarium oxysporum* N17B

Band No.[a]	R_f values[a]	Rat oral toxicity[b]	Cytotoxicity (LC_{50} in mg equiv. of fungus-infested rice extracted/mL)[c]			
			3T3 mouse fibroblasts	GM 498 human fibroblasts	HeLa cells	Rat beating heart cells
Control (40% ethanol)		-	-	-	-	-
Bands 1–8	0.16–0.69	-	-	-	-	-
Band 9 (yellow)	0.76	+, 1 D, HI, HS, HH	35	50	35	50
Band 10	0.83	-	-	-	-	-
Purified wortmannin[d]	0.78	+, D, HI, HS, HH	50	-	-	-

[a] TLC plates were developed in chloroform–methanol (97 + 3), and fractions were detected by spraying guide strips with 20% H_2SO_4 in methanol or with acidic *p*-anisaldehyde.
[b] Toxicity of extracts 16 h after feeding the equivalent to 2.5 g fungus-infested rice dissolved in 0.5 mL 40% ethanol to groups of 3 rats by gastric intubation. Abbreviations: + = definite toxic effect; - = no detectable toxic effect; D = death; number = number of dead rats; HI = intestinal hemorrhage; HS = hemorrhage in stomach; and HH = hemorrhage in heart.
[c] - = cytotoxicity > 50 mg equivalent of fungus-infested rice extracted/mL.
[d] Lethal dosages at 50, 25, and 4 mg/kg caused hemorrhage in tissues.

20 mg equivalents/mL in 3T3, 50 mg equivalents/mL in GM498, 20 mg equivalents/mL in HeLa cells, and 50 mg equivalents/mL in beating heart cells) (Table 4).

Florisil column Fraction 2 was further fractionated by TLC on silica gel to yield 10 fractions based on migration distance. All of the toxicity was present in a yellow band (Band 9), which caused the major clinical symptoms of toxicity to rats described above, including death and hemorrhage in the intestine, stomach, and heart (Table 5). This fraction was also cytotoxic at high concentrations (LC_{50} values of 35 μg/mL in 3T3, 35 μg/mL in HeLa cells, 50 μg/mL in GM498, and 50 μg/mL in neonatal rat beating heart cells).

Toxicity of Purified Wortmannin

Purified wortmannin was obtained by crystallization of the yellow material in TLC Band 9 from ethanol. As reported previously (2), the crystalline material was demonstrated by physical and spectral properties to be wortmannin, which was also reported to be produced by *Penicillium wortmannii* (32, 33), *Penicillium funiculosum* (34), and *Myrothecium roridum* (35). Wortmannin caused death to rats at 25 and 50 mg/kg within 5 h of administration by gastric intubation. Death was accompanied by hemorrhage in the intestine and stomach. At 4 mg/kg, it caused death within 20 h of administration accompanied by hemorrhage in the intestine, stomach, and heart (Table 5). Also, wortmannin exhibited low degrees of cytotoxic activity (LC_{50} values of 50 μg/mL in 3T3, and >50 μg/mL in GM 498, HeLa cells, and neonatal rat beating heart cells).

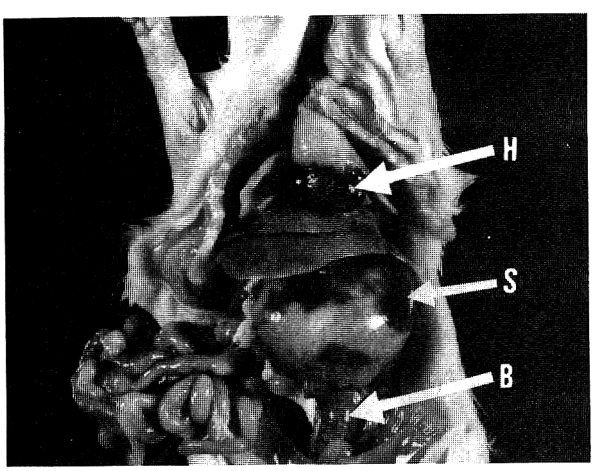

Figure 2. Rat intubated with toxic extract of rice medium on which *Fusarium oxysporum* N17B was grown. Note hemorrhage of stomach (S) and heart (H) and distension of urinary bladder (B) with bloody urine (hematuria), indicated by arrows.

Discussion

The procedure outlined in Figure 1 provides an effective and relatively simple procedure for the detection and purification of the hemorrhagic factor wortmannin from *F. oxysporum* grown on rice medium. These techniques can be used to detect production of the hemorrhagic factor by other *Fusarium* species cultured in rice medium and perhaps in field samples. The methodology uses inexpensive technology, including biological testing. It is relatively sensitive when compared to physical methods that do not differentiate between toxic and nontoxic components.

The procedure was designed to incorporate several features that facilitate its routine use. The materials are inexpensive and readily available. None of the steps requires the evaporation of water. Ethyl acetate was selected as the extracting solvent because it efficiently removes the toxic material from the culture substrate with little or no residue remaining, it is readily evaporated, and it constitutes much less of a disposal problem than chlorinated hydrocarbon solvents. The method introduces the use of Florisil column chromatography in wortmannin analysis and purification. Florisil allows much better recovery of the toxic material than does silica gel column chromatography (unpublished results). Overall, the procedure yields a highly purified (approximately 99% pure) crystalline product in good yield.

Currently, much interest exists in developing cell culture systems for toxicity testing because they have a number of advantages compared to whole animal testing. They are usually less expensive, use smaller sample sizes, are more quantitative and rapid, and avoid ethical considerations associated with whole animal testing. The cytotoxicity assays employed in this study possess these advantages, but it should be noted that the animal feeding or oral intubation studies provide different and complementary information (Figure 2). Many of the various toxic effects observed in whole animals (e.g., congestion and hemorrhaging of tissues) cannot be effectively modeled in cell culture. Ultimately, the final assessment of toxicity of a fungal extract or any other agent must be performed in live intact animals.

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MYCOTOXINS

Rapid Solvent-Efficient Method for Liquid Chromatographic Determination of Ochratoxin A in Corn, Barley, and Kidney: Collaborative Study

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A joint interlaboratory study of a rapid, solvent-efficient liquid chromatographic method for determination of ochratoxin A (OTA) in barley, corn, and pork kidney tissue was conducted by AOAC, the International Union of Pure and Applied Chemistry, and the Nordic Committee on Food Analysis in 16 laboratories in Europe, Canada, and the United States. OTA was added to barley and corn at 10, 20, and 50 ng/g and to kidney at 5, 10, and 20 ng/g. Duplicate test portions were prepared at 20 ng/g for corn and barley and 10 ng/g for kidney. Mean recoveries of OTA ranged from 53 to 97%. Within-laboratory relative standard deviations were 7.9, 20.1, and 15.7% for barley, corn, and kidney tissue, respectively. Between-laboratories relative standard deviations were 20.7–31.7% for all concentrations of OTA in barley and corn and 68.0, 41.8, and 32.7% for OTA concentrations of 5, 10, and 20 ng/g, respectively, in kidney. OTA identity was confirmed by methyl ester derivatization followed by liquid chromatography. The method has been adopted first action by AOAC as quantitative at the levels tested for OTA determination in corn and barley.

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The recommendation was approved by the General Referee and the Committee on Foods I and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1992) *J. AOAC Int.* 75, 223–225.
[1] Deceased.
This project was sponsored by the International Union of Pure and Applied Chemistry (IUPAC) Commission on Food Chemistry.

The 2 current AOAC thin-layer chromatographic (TLC) methods (1) for ochratoxin A (OTA), developed for the analysis of barley and green coffee, have been widely used for more than 20 years and applied to many different products, including animal tissue. Although many advances in technique and methodology have been made over the years, and numerous new methods have been developed (2), none of these methods have been studied collaboratively.

Gradually greater significance has been accorded OTA as a potential health hazard because of increased awareness of its occurrence in food, feed, edible animal tissues and serum, and even human serum. OTA's potential as a carcinogen led the International Agency for Research on Cancer (IARC) to include methods of analysis for the mycotoxin in its monograph of methods for carcinogens (3). Also, in 1985 the Commission on Food Chemistry of the International Union of Pure and Applied Chemistry (IUPAC) initiated a joint project with the AOAC for the collaborative study of an improved TLC/liquid chromatographic (LC) method (4) for OTA in barley, corn, and swine tissue. Advances in methodology, such as solid-phase extraction cleanup, and recognition of the need to combine rapid, economical, solvent-efficient procedures with quantitative LC or TLC led us to devise a new improved method. This method, which was applied to spiked barley, corn, oats, soybeans, and wheat before collaborative study, was validated independently in our laboratory by both LC and TLC, and in another laboratory by LC. Precision was adequate in all cases and recoveries ranged from 75 to 90%.

Barley, corn, and kidney were chosen for the collaborative study because OTA occurs naturally in all 3 materials. Among cold climate grains, barley contains the most background interferences; corn, a warm climate crop, contains intermediate levels of background interferences; and kidney, the animal organ in which OTA accumulates, is the tissue that has received the most attention (5).

The method consists of extraction of OTA from grain or kidney with chloroform–0.1M phosphoric acid (9 + 1), parti-

tion into bicarbonate, solid-phase extraction onto a 0.5 g C18 bonded silica matrix, acidification of the column, and elution with ethyl acetate–methanol–acetic acid (95 + 5 + 0.5). OTA is then determined by reversed-phase LC with fluorescence detection. Although TLC quantitation can also be used in the method, only LC was included in the collaborative study.

Invitations to collaborate were sent to 44 laboratories around the world that in the past had some involvement in OTA analysis. Of these, 19 agreed to participate, 11 in Europe (8 countries), 2 in Canada, and 6 in the United States. Results from 16 collaborators were returned. One collaborator withdrew because of insufficient time, and 2 others could not complete the study in time for this report. The results of the study are presented here.

Collaborative Study

Preparation of Test Portions

We obtained barley and corn from a local feed store and fresh pork kidneys from a meat market. These were ochratoxin-free as determined by repeated analyses using the method described below. Naturally contaminated grains and tissue were not available and were not used in this study. The kidneys were ground once in a Hobart meat grinder and blended by hand; 50 g portions were weighed into 120 mL screw-cap, polyethylene specimen cups and frozen to −20°C. The kidney test portions and the whole grains were packed with dry ice and shipped overnight to Isomedix Operations, Inc., NJ, for radiation (1.5 Mrd) to inactivate the microorganisms. Boxes containing the tissue and grains were irradiated, returned unopened, and received on the third day after they had been shipped. The tissue test portions were for the most part still frozen solid; however, some containers had been broken or cracked during shipment, and the tissue had to be transferred to new containers. Holes (3–4 mm) were drilled in the test portions of frozen kidney and the OTA spiking solution (100 μL) was added with a microsyringe. The cups were kept upright and returned to the freezer to let the solvent evaporate (overnight).

After irradiation, the barley and corn were ground in a rotary beater mill (Retch, SR-3) with a 1 mm screen and blended 30 min in a Hobart paddle blender set at the slowest speed. Test portions (50 g) of grain were then weighed into 120 mL cups as above. A small hole was made with a spatula, the OTA spiking solution (100 μL) was added, and the hole was closed after a short wait.

Preparation of Spiking Solutions

Dried film quantities of OTA, previously calibrated by UV spectrophotometry (UV) (dissolved in benzene–acetic acid (99 + 1)) according to AOAC [973.37C(i)] (1), were dissolved in chloroform–acetic acid (99 + 1) to ca 37 μg/mL and the OTA concentration was determined by UV at 333 nm [MW = 403, ε = 6192] (6). (The concentrations measured in the 2 solvent mixtures were 36.8 and 37.5 μg/mL for chloroform–acetic acid and benzene–acetic acid, respectively.) Aliquots of the chloroform–acetic acid solution were then diluted to the appropriate concentrations for spiking. The same volume (100 μL) of spiking solution was used for each 50 g test portion at each spiking level (Table 1).

Study Protocol

The protocol for this study was approved by the General Referee, the AOAC Committee on Foods I, and the consulting statistician. The levels of applicability were selected to comply with international regulatory limits and to meet requirements of surveys and programs for monitoring OTA in commodities and food products. Test portions were randomly numbered after they were spiked. All collaborators received identical sets of test portions; a set for each commodity consisted of a control, 4 test portions spiked at 3 levels (the middle level in duplicate), and a practice test portion of known concentration to be analyzed first. All test portions were to be quantitatively removed from their containers with the extraction solvent. The collaborators were supplied with a vial of dry film OTA standard (the same lot and preparation used for the spiking solutions); Supelclean LC-18, 3 mL solid-phase extraction tubes; supplementary instructions on using the tubes; instructions on handling the test portions; a report form; a packing list; and a copy of the method. The test portions, packed with 20 kg dry ice, were shipped by overnight delivery by commercial carrier. Most arrived in frozen condition. However, because of excessive delays at import terminals or in final delivery, test portions of kidney tissue in several of the European shipments could not be used. The collaborators were asked to confirm OTA identity by formation of the methyl ester derivative followed by LC analysis of 4 of the test portions, 1 of each commodity and 1 of their own choosing.

991.44 Ochratoxin A in Corn and Barley Liquid Chromatographic Method

First Action 1991

(Ochratoxin A causes kidney and liver damage and is carcinogenic in some animals. Observe precautions given in introductory statement of this chapter, and see safety notes on solvents specified.)

(Quantitative for ochratoxin A at ≥10 ng/g in corn and barley.)

Method Performance:

Corn, 10 ng ochratoxin A/g

$s_R = 1.7$; $RSD_R = 20.7\%$

Corn, 20 ng ochratoxin A/g

$s_r = 3.3$; $s_R = 4.6$; $RSD_r = 20.1\%$; $RSD_R = 28.4\%$

Corn, 50 ng ochratoxin A/g

$s_R = 12.2$; $RSD_R = 31.7\%$

Barley, 10 ng ochratoxin A/g

$s_R = 2.0$; $RSD_R = 27.2\%$

Barley, 20 ng ochratoxin A/g

$s_r = 1.1$; $s_R = 3.8$; $RSD_r = 7.9\%$; $RSD_R = 26.5\%$

Barley, 50 ng ochratoxin A/g

$s_R = 10.2$; $RSD_R = 27.6\%$

Table 1. Collaborative results (ng/g) for determination of OTA by LC with fluorescence detection

	Barley					Corn					Kidney				
Coll.	0	10	20	20[a]	50	0	10	20	20[a]	50	0	5	10	10[a]	20
1	1.0	10.6	20.0	20.3	47.8	1.8	11.1	21.1	21.1	50.7	—[b]	—[b]	—[b]	—[b]	—[b]
2	0	0.9	8.5[c]	17.5	42.0	4.5	9.0	10.0	15.0	41.0	—[b]	—[b]	—[b]	—[b]	—[b]
3	1.6	7.5	13.0	14.5	33.0	3.3	9.7	15.1	15.3	41.8	0.8	5.2	6.6	6.9	13.2
5	0	4.8	15.2	12.4	38.5	0	5.7	12.8	13.5	16.2	0	4.9	7.3	7.8	10.7
6	1.5	6.0	13.3	10.6	13.4	0.4	7.2	14.5	11.2	13.1	0[d]	0.5[d]	0.3[d]	0.8[d]	4.3[d]
7	3.6	10.0	16.4	17.6	45.7	0	6.9	20.2	15.2	38.5	0	2.2	5.9	4.8	10.2
10	0.4	4.3	7.2	7.4	21.3	0.5	6.8	14.4	11.8	—[e]	—[f]	—[f]	—[f]	—[f]	—[f]
11	0	9.8	21.1	0[c]	48.3	0	7.4	25.8	25.4	37.4	0	11.2	7.7	11.2	15.5
12[g]	0	0	13.6	12.8	15.2	0	3.2	9.6	16.0	32.8	0	0	0	2.8	2.0
14	0	7.2	13.0	12.0	36.1	0	6.0	12.7	12.3	32.5	0	1.2	4.1	3.2	4.8
15	0	6.6	13.2	15.0	37.8	0	7.0	15.2	11.4	32.2	—[b]	—[b]	—[b]	—[b]	—[b]
16	0	8.9	19.3	20.7	48.0	0	9.8	18.9	20.4	42.7	—[b]	—[b]	—[b]	—[b]	—[b]
17	0	6.8	16.7	17.1	39.6	0	8.8	14.7	17.8	44.3	0	3.2	6.5	5.5	8.1
18	3.2	6.3	17.2	17.6	44.1	0.8	10.1	15.2	27.0	55.9	—[b]	—[b]	—[b]	—[b]	—[b]
19	0	8.0	10.9	11.8	26.9	0	7.2	8.4	17.7	40.0	0	5.9	13.5	11.8	11.4
20	0.2	4.6	9.7	12.5	32.9	0.4	10.0	17.9	18.0	52.1	—[b]	—[b]	—[b]	—[b]	—[b]
Mean	0.8	7.4	—	14.4	37.0	0.8	8.2	—	16.3	38.5	0.2	4.4	—	6.9	11.4
Mean rec., %	—	74	—	72	74	—	82	—	82	77	—	97	—	73	53
s_r	—	—	—	1.1	—	—	—	—	3.3	—	—	—	—	1.2	—
s_R	1.4	2.0	—	3.8	10.2	1.6	1.7	—	4.6	12.2	0.7	3.3	—	3.1	3.5
RSD_r, %	—	—	—	7.9	—	—	—	—	20.1	—	—	—	—	15.7	—
RSD_R, %	—	27.2	—	26.5	27.6	—	20.7	—	28.4	31.7	—	68.0	—	41.8	32.7

[a] Blind duplicate.
[b] Data submitted but considered invalid because tissue thawed in transit.
[c] Excluded from statistical calculations as outlier based on Cochran and Grubbs tests.
[d] Excluded from statistical calculations because 3 of 4 values were <1.0 ng/g; see text.
[e] No determination because of temporary failure of liquid chromatograph.
[f] Collaborator 10 did not analyze tissue because recovery was only 3.2% for practice test portion.
[g] Data from Collaborator 12 excluded from statistical calculations; see text.

A. Principle

Ochratoxin A is extracted from grains with chloroform–aq. phosphoric acid, and isolated by liquid-liquid partitioning into aq. bicarbonate solution. The extract is applied to C18 column, and ochratoxin A is eluted with ethyl acetate–methanol–acetic acid. Ochratoxin A is identified by reversed-phase LC, and quantified by fluorescence. Chromatography of ochratoxin A methyl ester derivative confirms identity.

B. Apparatus

(a) *High-speed blender.*—1250 mL capacity jar with cover.

(b) *Liquid chromatograph.*—Pump, 0.5–5 mL/min, 3000 psi, flow reproducibility ±0.1%. Injection valve with 25 μL loop. For fluorescence detector with grating, set excitation at 333 nm and emission at 460 nm; for fluorescence detector with filter, use 420 nm cut-off filter. Use compatible recorder or integrator. Operating conditions: flow rate 1.0 mL/min; sensitivity set for 4–8% full scale response for 2.0 ng ochratoxin A and <2% noise; retention time 10–13 min; injection volume 20–25 μL, use 50 μL to fill 25 μL loop.

(c) *LC analytical column.*—250 × 4.6 mm id packed with 5 μm C18 bonded silica gel (Supelco Inc., Bellafonte, PA 16823, or equivalent).

(d) *Adsorption column.*—500 mg 40 μm C18 in 3 mL polypropylene tube (Supelco Inc., or equivalent).

(e) *Vacuum manifold.*—12-port, with stopcocks for each port for holding C18 columns.

(f) *Glass fiber filters.*—0.33 mm thickness, 1.5 μm pore retention, 9.0 cm diam. (Whatman No. 934 AH, or equivalent).

(g) *Microfilter.*—0.45 μm pore retention syringe filter (Gelman acrodisc 3CR PTFE, or equivalent).

C. Reagents

(a) *Solvents.*—Chloroform, methylene chloride, ethyl acetate, benzene, and methanol, ACS grade, in glass. Acetonitrile, LC grade. (*Caution*: Benzene is toxic, tumor-producing agent. Use in hood.)

(b) *Chemicals.*—Phosphoric acid, sodium bicarbonate, acetic acid, ACS grade.

(c) *Diatomaceous earth.*—See 973.37C(a).

(d) *LC mobile phase.*—Water–acetonitrile–acetic acid (99 + 99 + 2). Use LC grade water. Degas.

(e) *Phosphoric acid solution.*—0.1M. Dilute 5.75 g 85% phosphoric acid to 500 mL with water.

(f) *Ochratoxin A standard solutions.*—About 24 μg/mL in benzene–acetic acid (99 + 1). Determine concentration as in **973.37C(i)**. Dilute stock solution with benzene–acetic acid solution to obtain working standard solution (4 μg/mL).

(g) *BF3–methanol solution.*—14% BF_3. (*Caution*: Use in hood. Avoid contact with skin, eyes, and respiratory tract.)

D. Preparation of Sample

Prepare sample as in **977.16**.

E. Extraction

Weigh 50 g test portion into blender, add 25 mL 0.1M phosphoric acid and 250 mL chloroform, and blend 3 min at medium speed. Near end of blending add 10 g (45 mL) diatomaceous earth. Filter extract through glass fiber paper covered with ca 10 g diatomaceous earth on 9 cm Buchner funnel (or by gravity through 32 cm fluted paper). Collect >50 mL filtrate.

F. Partition

Transfer 50 mL filtrate to separatory funnel. Add 10 mL 3% sodium bicarbonate, and shake gently. Let phases separate. If emulsion forms, centrifuge 2 min at 2000 rpm. Collect upper (bicarbonate) phase for column extraction.

G. Column Preparation

Place C18 columns on vacuum manifold ports with 25 mL Erlenmeyer flasks or beakers inside manifold for collecting conditioning and wash solvents. Wash each column 2 times with ca 2 mL methanol, 2 mL water, and 2 mL 3% sodium bicarbonate. To speed elutions, apply gentle suction (or apply pressure using 5–10 mL syringe adapted to top of column). DO NOT LET COLUMN RUN DRY. Leave ca 2 mm solvent on top of frit.

H. Column Extraction

Pipet 5 mL bicarbonate extract to C18 column, followed with 2 mL 0.1M phosphoric acid, then 2 mL H_2O. Discard washes. Elute ochratoxin A with 8 mL ethyl acetate–methanol–acetic acid (95 + 5 + 0.5) into 10 mL test tube or vial containing 2 mL water. Used C18 column may be regenerated by conditioning as described for new columns. Shake or stir eluate with glass rod to mix 2 phases. Pipet ochratoxin A extract (upper phase) to 7 mL screw-cap vial. Rinse remaining upper phase from tube with 2 × 1 mL ethyl acetate and add to ochratoxin A. Evaporate extract just to dryness on steam bath under N_2.

I. LC Determination

(a) *Standard curve.*—Prepare standard curve at start of analysis and whenever chromatographic conditions change. Into separate 4–5 mL Teflon-lined, screw-cap vials, use μL syringes to dispense 25, 50, 100, and 200 μL working standard solution (4 ng/μL). Evaporate just to dryness under N_2. Add 1.00 mL LC mobile phase to each vial for final ochratoxin A concentrations of 2.5, 5, 10, and 20 ng/25 μL.

Chromatograph each standard solution. Using the origin as a fixed point, plot peak heights or areas vs 2.5, 5, 10, and 20 ng ochratoxin A for linearity. Calculate (normalize) response (R_{1-4}) of 1 ng ochratoxin A for each of the 4 standard concentrations (C_{1-4}). Calculate the average response (R_a) for 4 standard concentrations, and determine percent deviation (D) of individual values from average as follows.

$$R_a = (R_1/C_1 + R_2/C_2 + R_3/C_3 + R_4/C_4)/4$$

$$D = 100[(R/C) - R_a]/R_a$$

Deviation should be ≤5%. If D >5, omit value and recalculate R_a using 3 standards. If 3 values do not agree, rechromatograph standard solutions or prepare new standard solutions.

(b) *Sample.*—Dissolve sample extract from **H** in LC mobile phase (500 μL) and filter through 0.45 μm microfilter into 5 mL screw-cap vial. Chromatograph sample. Identify ochratoxin A from retention time (must be same as that of standard ochratoxin A). If sample ochratoxin A response is outside range of standard curve, adjust sample volume by concentrating or diluting sample solution. Reserve remaining sample solution for identity confirmation by formation of methyl ester. Calculate ochratoxin A concentration in sample as follows.

$$OTA\ (ng/g) = (R_s \times V_T)/(R_a \times V_I \times W) = (R_s \times F)/R_a$$

where W = (50 g × 50 mL × 5 mL)/(250 mL × 10 mL) = 5 g (weight of test sample represented by final extract); R_s = response of test sample injected; R_a = calculated average normalized responses (response for 1 ng ochratoxin A) of the 4 working standard solution concentrations; V_T = final test sample volume (500 μL); V_I = test sample injected (25 μL); F = 4.

J. Confirmation of Identity of Ochratoxin A by Methyl Ester Formation

Quantitatively transfer remaining reserved sample, **I(b)**, to 25 mL separatory funnel, using 3 × 1 mL methylene chloride to rinse vial. Shake and let layers separate. Collect lower layer into 5 mL vial and evaporate to dryness. Transfer 100 μL working standard ochratoxin A solution to another 5 mL vial and evaporate to dryness. Add 0.5 mL 14% BF_3–methanol to each vial, cap, and heat 15 min in 50–60°C water bath. Evaporate to dryness on steam bath under N_2. If H_2O is present, add 1 mL acetonitrile and continue evaporation to dryness. Cool and dilute with LC mobile phase to same volume as used for LC analysis. Chromatograph derivatized sample and standard. Positive confirmation is disappearance of peak at R_t for ochratoxin A (10–12 min) and appearance of new peak at same R_t as standard methyl ester of ochratoxin A (ca 15 min later). Careful quantitative preparation of ester can confirm quantitative analysis of ochratoxin A in sample, and should agree ±5%.

Ref.: JAOAC **75**, May/June issue (1992)

CAS-303-47-9 (ochratoxin A)

Table 2. Confirmation of identity of OTA by collaborators

Coll.	Ochratoxin A concn, ng/g		
	Barley	Corn	Pork kidney
1	10	20	10
3	0[a]	20	0[a], 10
5	20	20	5, 10
6	20, 50	20	—
7	20, 50	50	20
12	20, 50	50	20
16	10	20, 50	20
17	20	20, 50	20
18	50	0[a], 20	—
19	50	0[a]	5, 20
20	20, 20	10	—

[a] See text for meaning of confirmation of identity for "zero" concentrations of OTA.

Results and Discussion

Data were returned by 16 of the 19 collaborators and were statistically evaluated (Table 1). Average recoveries of OTA ranged from 72 to 82% for the grains and from 53 to 97% for kidney. The ranges of individual recoveries were barley 26.8–106%, corn 26–135%, and tissue 24–224%. Values for within-laboratory precision, RSD_r, for blind duplicates (20 ng/g grain, 10 ng/g tissue) were 7.9% for barley, 20.1% for corn, and 15.7% for tissue. Values for between-laboratories precision, RSD_R, were 20.7–31.7% for grains. For tissue, the RSD_R values ranged from 32.7% for the 20 ng/g level to 68.0% for the 5 ng/g level. Six collaborators reported low levels of OTA in the barley and corn controls, and 2 others reported a low level for either the barley or the corn control for a total of 14 false positives. The levels for the barley and corn false positive controls ranged from 0.2 to 4.5 ng/g with a mean of 0.8 ng/g for all the grain controls. One false positive in 7 tissue controls was reported.

Because our own thorough studies determined that the controls were devoid of any OTA, we believe that those collaborators reporting non-zero values were incorrect. The 95% confidence limits for the estimates of the control concentrations were computed. The limits (95%) for the barley and corn controls did not include zero. Therefore, for these 2 sets of controls, the true answer, zero, is not included within the confidence limits, and the discrepancies cannot be attributed to random fluctuations. Also, a Fisher's Exact test was performed to compare the proportion of collaborators who actually found a false positive vs the proportion of collaborators expected to find a false positive (namely, 0%). All 3 statistical tests gave a signal of significance ($p \leq 0.01$). The finding of a significant difference in these tests (confidence interval test, equivalent to a *t*-test, and the Exact test for a proportion) means that the probability of the results happening by chance alone is so improbable as to be considered impossible. Therefore, something other than chance must be operative, such as intrinsic method bias or matrix interferences.

The statistics shown in Table 1 were obtained after the data from Collaborator 12 were removed. These data were excluded because of low recoveries (0 and 30%, for 10 and 50 ng/g barley, respectively, and 32% for 10 ng/g corn, the 3 lowest recoveries reported for grain, and 0, 0, 28, and 10% for kidney) and because the chromatograms indicated that the LC conditions were uncontrolled.

OTA peaks were cleanly separated in some chromatograms, but appeared as shoulders on matrix peaks or were completely buried under background peaks in others. In addition, 1 of the replicate values reported by Collaborator 11 was identified as an outlier by the Cochran and Grubbs tests. No other values were excluded on the basis of these tests. However, the kidney data of Collaborator 6 were excluded because of uniformly low recoveries (10, 3, 8, and 22% for the 5, 10, 10, and 20 ng/g levels, respectively).

Six of the 11 European collaborators expressed reservation about the integrity of the kidney test portions that they received. The tissue had thawed and the containers were leaking as a result of delays in delivery, which took 1–10 days longer than the 4–5 days expected. Therefore, Collaborator 20 did not analyze the kidney test portions, and the data reported by the other 5 collaborators were omitted from this report. Collaborator 10 did not analyze the kidney test portions because the recovery for the practice test portion was only 3.2%.

Confirmation of OTA Identity

Eleven collaborators confirmed the identity of OTA in 4 test extracts of their own choosing (Table 2). Methyl ester derivatives were formed and chromatographed by LC. OTA identity was confirmed when a chromatogram of the esterified extract exhibited no OTA peak and a new peak appeared at the retention time of the OTA methyl ester. The identity of OTA was confirmed in test extracts that represented all spiking levels, with the largest number of confirmations occurring, as might be expected, for 20 ng/g, the OTA level in the blind duplicate test portions of grain and the highest OTA level in kidney tissue.

The confirmation procedure was applied to 4 control extracts. In 1 case, the collaborator applied the esterification procedure to the extract before determining the OTA concentration; no OTA was found. The other 3 cases are difficult to explain. One collaborator, who found 1.6 ng OTA/g in control barley, confirmed its identity and quantitated the methyl ester, finding the equivalent of 0.82 ng OTA/g. This collaborator also confirmed the identity of the OTA found at 0.8 ng/g in control kidney. The same collaborator quantitated OTA as the methyl ester for the 20 ng/g corn and 10 ng/g kidney test portions, finding the equivalent of 20.3 and 7.6 ng OTA/g, respectively. One collaborator did not perform confirmatory tests because the BF_3-methanol solution was unavailable; 4 others elected not to do them. One collaborator quantified the OTA methyl ester in 3 test extracts from barley and corn spiked at 50 ng/g and barley spiked at 20 ng/g, determining the equivalent of 51.2, 52, and 13.2 ng OTA/g, respectively. These values are in excellent agreement with the levels actually present and with

Table 3. LC columns used by collaborators

Stationary phase	Name	Size, μm	Length × id, mm		Source
Ultrasphere	ODS	5	250	4.6	Beckman, Fullerton, CA
μBondapak	C18	10	150	3.9	Waters, Milford, MA
Dynamax	C18	5	250	4.6	Rainin, Woburn, MA
Microsorb	ODS 2	5	250	4.6	Rainin, Woburn, MA
Hypersil	ODS	5	150	4.6	Shandon, Ashmoor, UK
Licrosorb	RP 18	5	150	4.6	E. Merck, Darmstadt, FRG
Lichrosorb	RP 18	5	250	4	E. Merck, Darmstadt, FRG
Lichrospher	RP 18	5	250	5	E. Merck, Darmstadt, FRG
Lichrospher	RP 18	5	150	4.6	E. Merck, Darmstadt, FRG
Spherisorb	ODS 2	3	100	4.6	Phase Separations, Queensferry, UK
Spherisorb	ODS 2	5	250	4.6	Phase Separations, Queensferry, UK
Spherisorb[a]	ODS 1	5	150	4.6	Phase Separations, Queensferry, UK
Supelcosil DB	C18	5	150	4.6	Supelco, Bellefonte, PA
Ultrasphere PTH	C18	5	250	4.6	Beckman, Fullerton, CA

[a] Non-end-capped C18 produced severe tailing of OTA peaks.

the results of the collaborator's own analyses. Another collaborator quantitated the OTA methyl ester for 1 of the spiked test portions (corn, 50 ng/g); the results were within ± 5% of the OTA values from the collaborator's own analysis.

Collaborators' Comments

One collaborator had difficulty in filtering tissue extracts; 2 others had difficulty in filtering both tissue extracts and grain extracts. All collaborators found it necessary to centrifuge the bicarbonate extracts. Collaborators suggested that the bicarbonate partition procedure specify gentle shaking for 30 s and that the 8 mL volume of eluant used for the solid-phase extraction be reduced to 2–3 mL. Another collaborator suggested that the use of an extraction solvent other than chloroform would be desirable. It was also pointed out that the use of closed stopcocks at the bottom of the solid-phase extraction tubes prevented dripping and possible loss of OTA.

Liquid Chromatography

The collaborators were asked to report the LC conditions they used. A great variety of reversed-phase columns (Table 3) worked well with the solvent system specified, except for 1 in which the packing material was not end-capped (Spherisorb, ODS 1). It produced severe tailing of the OTA chromatographic peaks. Many different fluorescence detectors were used, including both filter and grating instruments. Several collaborators pointed out that for fluorometers with filters, 418 nm cutoff filters should be used, whereas collaborators using grating spectrofluorometers determined that the emission monochromator should be set at 460 nm for maximum response, not 420 nm as was specified in the method sent to the collaborators. Six collaborators submitted their chromatograms. Except for those of Collaborator 12 as described above, all were clean chromatograms. Other collaborators commented that the chromatographic system worked well. Several had instrument or integrator problems and some used peak height measurement for quantitation. Several collaborators recommended using gradient LC or increasing the acetonitrile concentration of the mobile phase. Changing the composition of acetonitrile-water in the mobile phase from (1 + 1) to (70 + 30) reduced the retention time of the OTA methyl ester from 38 to 15 min (Spherisorb ODS 2).

Two collaborators calibrated the dry film OTA standard by UV spectrophotometry (dissolved in 4 mL mobile phase). Their results, 26 and 28.0 μg/mL, are about 94 and 102%, respectively, of the value obtained by the Associate Referee (27.6 μg/mL). Two collaborators observed that OTA standard stored in the mobile phase degraded with time. Detector response decreased about 5–10% in 1 week for the 300 and 600 μg/mL standards. Changes were observed even if solutions were refrigerated. However, for most of our work, we used an acetonitrile–phosphate buffer mobile phase and found OTA standard to be stable in this buffer for many months at room temperature. One would expect standard solutions containing acetic acid to be stable, but that question needs to be investigated further. Failure to calibrate standards and standard instability may account for the variability of the recoveries obtained by collaborators in this study.

Recommendation

The Associate Referee recommends that the rapid solvent-efficient method evaluated in this collaborative study be adopted first action for determination of OTA at ≥ 10 ng/g barley or corn.

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OILS AND FATS

Capillary Column Gas Chromatographic Method for Analysis of Encapsulated Fish Oils and Fish Oil Ethyl Esters: Collaborative Study

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A gas chromatographic (GC) method using a capillary column for analysis of encapsulated fish oils and ethyl esters was studied collaboratively in 21 laboratories. Each collaborator analyzed 6 soft-gelatin encapsulated samples; 5 were triacylglycerol oils (one was a blind duplicate), and one was an ethyl ester concentrate of omega-3 (n-3) polyunsaturates. Constituent fatty acids of the oils were converted to methyl esters by base-catalyzed transesterification of the oils; any free acids in the oils were esterified by subsequent reaction with BF_3/CH_3OH. The ethyl ester concentrate required no further derivatization. Results were reported as area percentages of 24 analytes of nutritional or biochemical interest. In addition, weights (mg/g sample) of EPA (all-*cis*-5,8,11,14,17-eicosapentaenoic acid or 20:5n-3) and DHA (all-*cis*-4,7,10,13,16,19-docosahexaenoic acid or 22:6n-3) were determined through the use of the internal standards, respectively, methyl tricosanoate (23:0) and ethyl 23:0, for the methyl and ethyl esters. The only instrumentation specifically required was a flexible fused silica capillary GC column coated with a bonded polyglycol such as Carbowax-20M, an oxygen scrubber installed in the carrier gas supply line, and a flame ionization detector (FID). Most of the collaborators experienced little difficulty in applying the method, and, of 2526 values reported, only 4.3% were identified as outlier values. The reproducibility relative standard deviations (RSD_R) compared favorably in most instances with, or were substantially better than, those of 2 earlier collaborative studies of fish oils. Because the variances were homogeneous, standard deviations and relative standard deviations determined on the area percent analyses of the blind duplicate oils were pooled to give the following mean values: s_r = 0.15, RSD_r = 4.88%, s_R = 0.41, and RSD_R = 12.91%. Analytes that rarely occur at greater than 0.5% in marine oils (22:0, 22:4n-6, 22:5n-6, 24:0, and 24:1) were not included in these calculations. The method was adopted first action by AOAC International as an American Oil Chemists' Society (AOCS)-AOAC method.

Current consumer interest in fish oils and related preparations sold as over-the-counter nutritional supplements (1) creates a need for accurate labeling of the products for the active ingredients, ostensibly EPA (all-*cis*-5,8,11,14,17-eicosapentaenoic acid or 20:5n-3) and DHA (all-*cis*-4,7,10,13,16,19-docosahexaenoic acid or 22:6n-3). The information needed for this labeling requires the application of the most appropriate fatty acid technology, given the complex chemical composition of fish oils and their derivatives.

Packed column gas chromatography (GC) is suitable for the analysis of most vegetable oils, but these columns lack the resolution necessary to separate the 60–80 fatty acids commonly present in fish oils. Therefore, capillary column GC, which has been used in marine lipid research for 2 decades, was deemed more suitable for quality control of commercial products. Improved separation of all components by capillary column GC

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The recommendation was approved by the General Referee and the Committee on Foods I and was adopted by the Official Methods Board of AOAC. See "Changes in Official Methods of Analysis" (1992) *J. AOAC Int.* 75, 223–225.
Mention of trade names, commercial firms, or specific products or instrumentation is for identification purposes only and does not constitute endorsement by the National Marine Fisheries Service.
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simultaneously improves quantitation in area percentages of EPA and DHA by the GC electronic integrator.

Although the results of GC analyses of fatty acid methyl esters are usually reported as area percentages of eluted components, the area percentages of EPA and DHA must be converted to absolute weights per gram of sample for informative labeling of nutritional supplements. This conversion requires the use of internal or external standards, and for accuracy, correction factors for the flame ionization detector (FID) response must be applied.

The purpose of the present international collaborative study was 2-fold. The first was to assess the effectiveness of polyglycol-coated capillary columns in separating fish oil fatty acids (as methyl and ethyl esters) of major nutritional or biochemical importance. The second purpose was to test the suitability of methyl and ethyl 23:0 as internal standards in the calculation of the absolute weights of EPA and DHA in fish oils and ethyl esters derived from that source.

Collaborative Study

Twenty-one laboratories participated in the study. Each collaborator received 3 capsules, each of 5 fish oils (including a blind duplicate of 1 oil) and 1 ethyl ester concentrate of n-3 polyunsaturates. Methyl and ethyl 23:0 were supplied as internal standards. The participants were instructed to use 2 capsules of each sample for analyst familiarization and instrument optimization and to submit results of a single analysis of the third capsule of each sample. Included with instructions, the study protocol, and the required calculations were 2 data report forms. The first provided space for a description of instrumentation, column dimensions and history, and operating parameters. The second was for listing the area percentages of 24 analytes of particular interest and the calculated weights (mg/g sample) of EPA and DHA in the 6 samples. A chromatogram of methyl esters of commercially encapsulated cod liver oil was included as an aid to peak identification and as an indicator of the resolution that could be expected from a properly operated polyglycol capillary column. The resolution of methyl docosapentanoate (22:5n-3) and methyl DHA should be at least 4.

991.39 Fatty Acids In Encapsulated Fish Oils and Fish Oil Methyl and Ethyl Esters—Gas Chromatographic Method

First Action 1991

AOCS–AOAC Method

Method Performance:
See Table **991.39** for method performance data.

A. Principle

Samples are weighed into Teflon-lined screw-cap glass tubes that contain appropriate internal standards. Fatty acids of oil samples are derivatized to methyl esters; ethyl ester samples require no derivatization. Prepared samples are analyzed by GC instrument equipped with fused silica column coated with bonded polyglycol liquid phase, oxygen scrubber in carrier gas line, and flame ionization detector. Method determines area percentages of 24 fatty acids and absolute weights (mg/g sample) of EPA (all-*cis*-5,8,11,14,17-eicosapentaenoic acid or 20:5n-3) and DHA (all-*cis*-4,7,10,13,16,19-docosahexaenoic acid or 22:6n-3).

B. Apparatus

(a) *Gas chromatograph.*—With flame ionization detector, capillary column injection system (split mode preferred at split ratio of 1:50), and suitable data processor. (*Note*: In fish oil analyses, samples are usually sufficient to permit operation in split mode.) Operating conditions: temperatures—injection port 250°; detector 270°; oven programmed from 170 to 225° at 1°/min (no initial or final hold). Helium or hydrogen carrier gas (99.99% pure, or better) with oxygen scrubber in line.

(b) *GC column.*—Fused silica, 30 m × 0.25 mm (or 0.32 mm) coated with bonded polyglycol, based on Carbowax-20M (e.g., SUPELCOWAX-10, or equivalent column that provides same elution pattern as that illustrated in Fig. **991.39** and baseline separation of 21:5n-3, 23:0, and 22:4n-6).

(c) *Constant temperature water bath.*—Maintained at 100°. Dry heater block may be used.

(d) *Glass tubes.*—16 × 125 mm. With leak-tight, Teflon-lined screw caps.

(e) *Vials.*—2 mL, with screw cap or crimp cap (for autosampler).

(f) *Analytical balance.*—Accurate to ±0.0001 g.

(g) *Dry nitrogen source.*

(h) *Glassware.*—Volumetric flasks, 25 and 100 mL; volumetric pipets, 1 and 2 mL; Pasteur pipets.

C. Reagents

(a) *Boron trifluoride.*—BF_3, 12% in methanol. Two mL amber glass ampoules (Supelco, Inc., Cat. No. 3-3020, or equivalent reagent, sealed in amber glass ampoules for extended shelf life). (*Caution*: BF_3 in methanol is a corrosive reagent and must be handled with care. Avoid eye and skin contact by use of protective shield and rubber gloves. Use only in properly operating fume hood.)

(b) *23:0 Methyl and ethyl esters.*—Reagents of 99+% purity as determined by TLC and GC analyses [Nu Chek Prep, Inc., Elysian, MN, Cat. No. N-23-M (methyl ester) and Cat. No. N-23-E (ethyl ester), or equivalent]. [**Note**: On request, the Charleston Laboratory, Southeast Fisheries Center, National Marine Fisheries Service, PO Box 12607, Charleston, SC 29422-0607, will provide capsules of collaborative study Sample 1 (steam-deodorized menhaden oil) for use in optimizing GC equipment.]

(c) *Reagent grade chemicals.*—Sodium hydroxide, methanol, isooctane, sodium chloride. (*Caution*: See safety notes on sodium hydroxide, methanol, and isooctane in Appendix, *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA.)

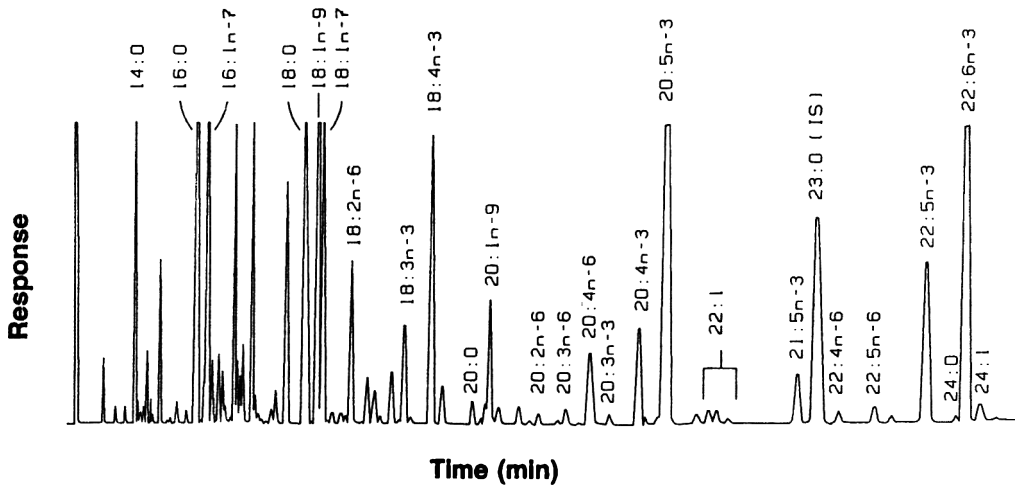

Figure 991.39. Temperature-programmed GC separation of menhaden oil fatty acid methyl esters on flexible fused silica column coated with bonded Carbowax 20M.

D. Preparation of Solutions

(a) *Alcoholic sodium hydroxide.*—0.5N. Dissolve 2.0 g NaOH in methanol and dilute to 100 mL with methanol.

(b) *Sodium chloride.*—Saturated solution. Dissolve 36 g NaCl in 100 mL H_2O.

E. Preparation of Standards

Accurately weigh ca 25 mg (± 0.1 mg) of 23:0 methyl or ethyl ester internal standard (IS) into 25 mL volumetric flask and dilute to volume with isooctane. Pipet 1.0 mL portions into screw-cap glass tubes and evaporate solvent in gentle stream of nitrogen. Store tubes in freezer if not to be used immediately.

F. Sample Preparation and Analysis

(a) *Oils.*—(*Note*: "Oil" applies to all encapsulated materials, including nonesterified fatty acids, with exception of ethyl esters.)

Accurately weigh ca 25 mg (±0.1 mg) oil into glass tube containing methyl ester IS, E. Add 1.5 mL 0.5N methanolic NaOH, blanket with nitrogen, cap, mix, and heat 5 min at 100°. Cool, add 2 mL BF_3 in methanol, C(a), blanket with nitrogen, cap tightly, mix, and heat 30 min at 100°. Cool mixture to 30–40°, add 1 mL isooctane, blanket with nitrogen, cap, and shake vigorously for 30 s while still warm.

Immediately add 5 mL saturated NaCl solution, blanket with nitrogen, cap, and agitate thoroughly. Cool to room temperature. When isooctane layer separates from aqueous lower phase, transfer isooctane layer to a clean glass tube, blanket with nitrogen, and cap.

Extract aqueous lower phase a second time with an additional 1 mL isooctane. Combine isooctane extracts and concentrate to ca 1 mL in stream of dry nitrogen.

Inject 1–2 µL into GC system.

(b) *Ethyl esters.*—Accurately weigh ≤15 mg (±0.1 mg) ethyl ester (usually more concentrated) into glass tube containing appropriate ester IS, E. Add 1 mL isooctane, blanket with nitrogen, cap, and mix thoroughly.

Inject 1–2 µL into GC system. If peak height of IS is ≤0.5 that of EPA or DHA peak, repeat analysis, using 2.0 mL IS.

G. Calculations

(a) *Area percentage.*—Calculate area percentages of fatty acid methyl esters or ethyl esters as follows:

$$\text{Area \% fatty acid}_X = [A_X/(A_T - A_{IS})] \times 100$$

where A_X = area counts of methyl or ethyl ester X; A_T = total area counts for chromatogram; and A_{IS} = area counts of IS.

(b) *Weight of EPA and DHA in oils.*—Calculate EPA or DHA, mg/g oil, as follows:

$$\text{EPA or DHA, mg/g} = [(A_X \times W_{IS} \times CF_X)/(A_{IS} \times W_S \times 1.04)] \times 1000$$

where A_X = area counts of EPA or DHA; A_{IS} = area counts of internal standard; CF_X = theoretical detector correction factor for EPA or DHA (0.99 for EPA, 0.97 for DHA); W_{IS} = weight of IS added to sample, mg; W_S = sample weight, mg; and 1.04 is factor necessary to express result as mg fatty acid/g oil (rather than as methyl ester).

(c) *Weight of EPA and DHA in ethyl esters.*—Calculate EPA or DHA, mg/g esters, as follows:

$$\text{EPA or DHA, mg/g} = [(A_X \times W_{IS} \times CF_X)/(A_{IS} \times W_S \times 1.08)] \times 1000$$

where terms are same as in (b), except use 1.08, factor necessary to express result as mg fatty acid/g ethyl ester (rather than as ethyl ester).

Ref.: AOCS Official Method Ce 1b-89. JAOAC 75, May/June issue (1992)

Table 991.39. Method performance for 991.39, fatty acids in encapsulated fish oils and ethyl esters[a]

Fatty acid	s_R	RSD_R, %	s_r	RSD_r, %	Fatty acid	s_R	RSD_R, %
	Fish oils, area %					Ethyl ester concentrate, area %	
14:0	0.58–0.98	8.31–13.12	0.49	5.8	14:0	0.06	17.07
16:0	0.44–1.91	5.66–10.02	0.54	2.9	16:0	0.11	12.24
16:1	0.55–2.59	6.80–26.73	0.51	4.3	16:1	0.10	30.96
18:0	0.05–0.42	3.05–14.43	0.06	1.8	18:0	0.15	7.00
18:1	0.23–0.68	1.92–6.71	0.19	1.6	18:1	0.50	5.72
18:2n-6	0.05–0.13	2.37–10.69	0.01	1.2	18:2n-6	0.06	7.93
18:3n-3	0.04–0.17	3.86–23.14	0.04	5.6	18:3n-3	0.08	12.85
18:4n-3	0.07–0.24	2.43–6.30	0.04	1.5	18:4n-3	0.15	7.62
20:0	0.02–0.17	7.78–84.25	0.01	5.5	20:0	0.03	10.12
20:1	0.15–0.45	4.46–18.13	0.13	7.3	20:1	0.45	3.19
20:2n-6	0.01–0.08	10.29–25.77	0.01	8.6	20:2n-6	0.10	32.42
20:3n-6	0.03–0.07	16.08–67.73	0.03	17.4	20:3n-6	0.11	35.22
20:3n-3	0.04–0.07	27.47–47.93	0.01	3.1	20:3n-3	0.07	29.32
20:4n-6	0.07–0.19	8.86–43.83	0.02	2.5	20:4n-6	0.09	7.37
20:4n-3	0.06–0.15	4.91–13.77	0.04	3.5	20:4n-3	0.15	7.86
20:5n-3	0.43–2.06	5.48–9.78	0.25	1.9	20:5n-3	1.54	5.83
22:0	0.02–0.38	11.77–141.42	0.02	8.9	22:0	0.07	73.94
22:1	0.14–0.80	8.88–17.24	0.11	7.9	22:1	0.50	4.68
22:4n-6	0.08–0.17	38.29–93.55	0.06	26.3	22:4n-6	0.41	84.99
22:5n-6	0.04–0.08	13.14–50.60	0.03	18.6	22:5n-6	0.14	43.08
22:5n-3	0.10–0.32	8.84–16.20	0.13	6.7	22:5n-3	0.36	8.52
22:6n-3	0.69–1.44	7.50–16.09	0.29	3.7	22:6n-3	1.40	7.51
24:0	0.02–0.14	48.38–100.00	0.01	10.9	24:0	0.11	158.70
24:1	0.07–0.73	41.22–102.20	0.03	7.4	24:1	0.23	35.16
	Fish oils, absolute weight (mg/g sample)					Ethyl ester concentrate, absolute weight (mg/g sample)	
20:5n-3	2.98–31.10	5.38–19.75	7.17	5.9	20:5n-3	20.40	9.15
22:6n-3	2.60–13.27	4.24–12.60	3.68	5.3	22:6n-3	14.15	8.97

[a] s_R and RSD_R for fish oils are ranges of values obtained in the collaborative study of 4 different fish oils. RSD_R values are elevated for analytes that rarely exceed 0.1–0.2% of total analytes (20:0, 20:3n-6, 22:0, 22:4n-6, 22:5n-6, 24:0, and 24:1).

Results

Details of equipment and some of the operating parameters used by the collaborators are listed in Table 1. All of the collaborators used helium carrier gas, with one exception; Collaborator 18 used hydrogen. Most of the columns used had been in operation for 1–10 months before the study began, but a number of collaborators reported the use of new columns, and one, Collaborator 10, reported that the column had been in service for 5 years. All but Collaborators 10, 15, 16, and 17 operated in split mode; split ratios varied from 1:50 to 1:100.

No restrictions were placed on the selection of GC instrumentation by the study participants beyond the mandatory use of a flexible fused silica capillary column coated with a bonded polyglycol liquid phase, an oxygen scrubber in the carrier gas line to protect the column, and a flame ionization detector. However, Collaborators 17 and 21 used columns coated with DB-225, which is not a polyglycol but is, rather, a liquid phase composed of 50% cyanopropylphenyl and 50% methyl silicone. Consequently, these collaborators were considered to be "procedural deviates" (2), and their data were not used in the statistical calculations.

The raw data submitted by the collaborators for the 6 samples and the statistical calculations of reproducibility between laboratories are listed in Tables 2–7. Collaborator 14 submitted a typewritten table of results for the 6 samples. Reported area percentages for Samples 1 and 2 were virtually identical, although Sample 1 was steam-deodorized menhaden oil and Sample 2 was the ethyl ester n-3 concentrate. However, the calculated weight data reported for EPA and DHA in Sample 2 were almost twice as great as those reported for Sample 1. This indicates that Collaborator 14 did, in fact, properly analyze Sample 2 but inadvertently submitted erroneous area percent data for this sample. Despite 2 requests, no correct report on Sample 2 was received from this collaborator.

Collaborator 18 initially reported the weights of EPA and DHA as ranging from 0.06 to 0.216 mg/g in the 6 samples. When contacted and asked to verify these data, the collab-

Table 1. Instrumentation and operating parameters used by collaborative study participants

Laboratory	GC	Column		Temperatures,°C			Program
		Liquid phase	Dimensions	Inj. port	Detector	Column	
1	HP-5880[a]	Carbowax-20M	25 m × 0.20 mm	250	270	170–225	1°/min
2	HP-5880	Carbowax-20M	50 m × 0.25 mm	275	300	193–225	hold 35 min then 1°/min to 225°
3	HP	DB-Wax	30 m × 0.25 mm	225	270	140–240	hold 2 min then 4°/min to 240°
4	PE-8420[b]	SUPELCOWAX-10	30 m × 0.32 mm	250	270	190–240	hold 8 min then 3°/min to 240°
5	HP-5880	SUPELCOWAX-10	30 m × 0.32 mm	250	270	170–225	1°/min
6	HP-5890	SUPELCOWAX-10	30 m × 0.32 mm	250	270	170–225	1°/min
7	HP-5840	SUPELCOWAX-10	30 m × 0.25 mm	250	270	170–225	1°min
8	HP-5790	DB-Wax	30 m × 0.32 mm	200	250	100–230	hold 2 min then 20°/min to 180° then 2°/min to 230°
9	HP-5880	SUPELCOWAX-10	30 m × 0.32 mm	250	270	170–225	1.5°/min
10	HP	SUPELCOWAX-10	30 m × 0.32 mm	250	270	170–225	1°/min
11	HP	DB-Wax	30 m × 0.32 mm	240	260	170–250	hold 2 min (no rate given)
12	HP-5880	SUPELCOWAX-10	30 m × 0.32 mm	250	270	170–225	1°/min
13	Carlo Erba	SUPELCOWAX-10	30 m × 0.32 mm	250	270	170–245	hold 30 s then 5°/min to 245°
14	Carlo Erba V6000	CP-Wax 58	50 m × 0.25 mm	250	270	190–210	hold 15 min then 1°/min to 210°
15	PE	SUPELCOWAX-10	60 m × 0.32 mm	250	270	180–230	1.5°/min
16	HP	SUPELCOWAX-10	30 m × 0.32 mm	250	270	170–225	1°/min
17	PE-8420	DB-225	30 m × 0.32 mm	30	270	30–200	30°/min to 120°, hold 5 min, then 5°/min to 200°
18	Varian	SUPELCOWAX-10	30 m × 0.32 mm	250	270	170–225	1°/min
19	HP-5890	DB-Wax	30 m × 0.32 mm	250	270	170–225	1°/min
20	PE-8500	SUPELCOWAX-10	30 m × 0.32 mm	250	250	185–205	hold 15 min then 2°/min to 205°
21	Shimadzu	DB-225	30 m × 0.25 mm	275	275	180–236	2°/min to 236°, hold 9 min

[a] Hewlett-Packard.
[b] Perkin-Elmer.

Table 2. Collaborative study results of analysis of Sample 1 (steam-deodorized menhaden oil)

	Composition, area %																								
	Laboratory																								
Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17[e]	18	19	20	21[e]	Mean	s_R[b]	RSD_R, %[c]	R[d]	Outliers/ No. labs.
14:0	7.6	8.2	8.0	9.0	6.3	8.3	7.4	8.0	8.6	8.4	7.3	8.3	8.7	8.0	10.1	8.1	5.3	10.7	9.2	8.5	7.8	8.34	0.98	11.70	2.7	0/19
16:0	18.0	18.9	19.0	20.0	15.8	18.8	18.0	18.5	20.0	19.4	15.4	18.6	19.4	18.7	20.5	19.0	8.6	24.4[e]	19.7	18.7	18.8	18.69	1.31	6.98	3.7	1/19
16:1	11.4	11.5	11.6	12.3	9.7	11.4	11.2	12.4	12.2	11.8	10.3	10.9	11.9	11.8	12.8	15.9	16.9	16.6	12.2	11.4	10.8	12.06	1.65	13.64	4.6	0/19
18:0	3.1	3.2	3.2	2.9	3.1	3.0	3.1	3.0	3.1	3.1	3.4	3.1	3.1	3.1	2.5[e]	3.2	2.4	3.7[e]	3.0	3.0	3.1	3.10	0.11	3.60	0.3	2/19
18:1	12.0	12.2	12.2	11.6	12.0	11.6	11.9	11.8	12.3	11.9	12.1	11.9	12.0	11.9	10.5[e]	12.2	10.2	14.4[e]	11.7	11.5	11.8	11.92	0.23	1.92	0.6	2/19
18:2n-6	1.2	1.2	1.2	1.1	1.2	1.2	1.2	1.2	1.2	1.2	1.3	1.2	1.2	1.2	1.0	1.2	3.3	1.5[e]	1.2	1.1	1.2	1.18	0.07	5.57	0.2	1/19
18:3n-3	0.8	0.8	0.8	0.7	0.8	0.8	0.8	0.8	0.8	0.8	0.3[e]	0.8	0.5[e]	0.8	0.7	0.8	1.6	1.1[e]	0.8	0.8	0.8	0.79	0.04	4.88	0.1	3/19
18:4n-3	2.9	3.0	3.0	2.7	3.0	2.9	2.9	2.9	2.9	2.9	3.3	2.9	2.9	2.9	2.5[e]	3.6[e]	3.3	3.6[e]	2.9	2.8	2.8	2.90	0.07	2.43	0.2	3/19
20:0	0.2	—[f]	0.2	0.2	—	—	0.2	0.2	—	0.2	0.9	0.2	0.2	0.2	—	0.2	1.5[e]	0.5[e]	0.2	—	0.2	0.21	0.02	7.78	0.0	2/13
20:1	2.0	1.6	2.0	1.7	2.4[e]	1.7	1.9	1.9	1.7	1.8	2.1	1.9	1.9	1.8	1.2[e]	1.8	1.0	1.6	1.7	1.8	1.9	1.81	0.15	8.04	0.4	2/19
20:2n-6	0.2	—	0.1	0.1	0.2	0.2	0.2	0.2	—	0.2	—	0.2	0.1	0.1	—	—	0.3	—	0.1	0.3	0.2	0.14	0.04	27.71	0.1	0/14
20:3n-6	0.2	—	0.1	0.2	0.3	0.2	0.2	0.2	—	0.2	—	0.2	0.2	0.2	0.2	0.2	0.4	—	0.1	0.3	0.2	0.18	0.06	40.63	0.2	0/16
20:3n-3	—	—	0.1	0.2	0.3	0.2	0.2	0.2	—	0.1	0.2	—	0.1	0.1	0.1	0.9[e]	0.9	—	0.1	0.1	0.1	0.15	0.05	41.73	0.1	1/14
20:4n-6	0.8	0.9	0.9	0.7	1.0	0.9	0.9	0.8	0.8	0.8	1.0	0.9	0.9	0.8	0.6	—	0.2[e]	0.9	0.8	0.8	0.8	0.84	0.09	10.52	0.2	1/19
20:4n-3	1.3	1.3	1.3	1.2	1.5	1.3	1.3	1.3	1.2	1.3	1.6[e]	1.3	1.2	1.3	0.8[e]	1.3	0.4	1.3	1.2	1.2	1.4	1.29	0.06	4.91	0.2	2/19
20:5n-3	14.7	14.6	14.0	13.2	15.5	13.4	14.3	13.0	14.7	12.6	13.3	14.0	13.7	13.7	10.0[e]	14.5	12.8	13.5	12.9	12.8	14.1	13.79	0.79	5.76	2.2	1/19
22:0	0.5	—	0.2	0.3	—	—	0.2	0.2	—	0.1	0.1	—	0.1	0.2	—	—	0.6	—	0.2	—	0.1	0.19	0.13	69.73	0.4	0/12
22:1	1.0	1.4	1.5	1.5	1.8	1.3	1.4	1.3	1.2	1.5	1.9	1.4	1.3	1.6	1.3	0.2[e]	0.7	—	1.2	1.9	0.9	1.43	0.25	17.24	0.7	1/18
22:4n-6	—	—	0.1	0.5	0.3	0.3	0.2	0.2	—	0.2	0.1	0.1	0.1	0.1	—	0.7	1.2	—	0.1	0.3	0.1	0.23	0.17	75.55	0.5	0/14
22:5n-6	0.2	—	0.2	0.3	0.3	0.2	0.2	0.2	—	0.1	0.2	—	0.1	0.1	—	—	0.6	—	0.1	0.3	—	0.17	0.08	45.59	0.2	0/14
22:5n-3	2.4	2.3	2.0	1.9	2.4	2.0	2.2	1.9	2.1	1.8	2.5	2.2	1.9	1.9	0.7	2.2	0.3[e]	1.2	1.8	2.1	2.2	2.05	0.29	14.59	0.8	1/19
22:6n-3	9.3	9.0	8.1	7.9	9.7	7.7	8.6	7.5	8.7	7.2	8.9	8.5	8.0	7.7	4.9	8.5	8.9	4.9	6.9	7.6	8.8	7.87	1.27	16.09	3.5	0/19
24:0	—	—	—	—	—	—	0.1	0.1	0.2	0.1	0.1	—	—	0.1	—	0.6	0.8	—	—	—	0.3	0.10	0.07	69.96	0.2	0/09
24:1	0.3	—	0.1	—	0.5	0.2	0.4	0.4	0.4	0.2	0.5	0.6	0.1	0.3	0.4	0.3	0.7	—	0.3	0.4	0.3	0.33	0.14	42.42	0.4	0/16
	mg/g sample																								
20:5n-3	105.0	101.2	119.5	119.1	111.7	117.0	111.6	118.1	114.0	119.7	118.0	189.4[e]	109.5	116.0	130.1	114.0	97.9	—	125.0	110.8	—	115.35	6.95	6.04	19.4	1/18
22:6n-3	65.0	61.2	67.9	71.1	67.4	66.4	66.3	68.0	66.0	67.4	71.0	112.0[e]	62.6	63.3	61.8	66.0	66.7	—	65.7	64.7	—	65.99	2.80	4.24	7.8	1/18

[a] Polyglycol column not used. Data are not included in statistical calculations.
[b] Reproducibility standard deviation.
[c] Reproducibility relative standard deviation.
[d] Reproducibility value ($2.8 \times s_R$).
[e] Outlier value, determined by Dixon and/or Grubbs tests, not used in statistical calculations.
[f] Denotes a value reported as "—," "< 0.05," "0," or "tr."

Table 3. Collaborative study results of analysis of Sample 2 (SuperEPA 500®, ethyl ester)

	Composition, area %																									
	Laboratory																									
Fatty acids	1	2	3	4	5	6	7	8	9	10	11	12	13	14[a]	15	16	17[a]	18	19	20	21[a]	Mean	s_R[b]	RSD_R, %[c]	R[d]	Outliers/ No. labs.
14:0	0.3	0.4	0.3	0.4	0.3	0.3	0.3	0.4	0.3	0.4	0.4	0.4	0.4	—	0.5	0.4	0.3	0.8[e]	0.5	0.5	0.4	0.38	0.06	17.07	0.2	1/18
16:0	0.9	0.9	0.9	0.8	0.7	0.8	0.8	0.9	0.9	1.0	1.1	0.9	0.9	—	1.1	1.0	0.6	1.9[e]	1.0	1.1	0.8	0.92	0.11	12.24	0.3	1/18
16:1	0.4	0.2	0.2	0.3	0.2	—	0.2	0.3	0.3	0.3	0.3	—	0.2	—	0.5	0.4	1.1[e]	0.8	0.3	0.5	0.2	0.31	0.10	30.96	0.3	1/16
18:0	2.0	2.2	2.2	2.0	2.0	2.1	2.0	2.1	2.0	2.3	2.5	2.2	2.2	—	2.0	2.2	1.8	3.0[e]	2.4	2.0	2.0	2.14	0.15	7.00	0.4	1/18
18:1	8.8	8.7	8.7	8.8	7.9	8.2	8.0	8.8	8.3	9.3	9.8	8.4	9.1	—	8.7	8.6	8.1	13.3[e]	9.4	8.5	8.3	8.69	0.50	5.72	1.4	1/18
18:2n-6	0.7	0.8	0.7	0.7	0.7	0.7	0.7	0.8	0.7	0.8	0.9	0.7	0.8	—	0.8	0.8	0.9	1.2[e]	0.8	0.8	0.8	0.76	0.06	7.93	0.2	1/18
18:3n-3	0.6	0.7	0.5	0.6	0.6	0.6	0.7	0.6	0.6	0.7	0.4	0.2[e]	0.6	—	0.7	0.6	1.0	1.0[e]	0.6	0.6	0.6	0.61	0.08	12.85	0.2	2/18
18:4n-3	1.9	1.9	1.9	1.8	1.7	1.8	1.7	1.8	2.0	2.0	2.2	1.9	1.9	—	1.9	2.2	1.4	3.0[e]	2.1	2.0	1.8	1.93	0.15	7.62	0.4	1/18
20:0	0.3	—[f]	0.3	0.3	—	—	0.3	0.3	0.3	0.3	0.8[e]	—	0.3	—	—	0.3	1.4[e]	0.4	0.3	0.5	0.3	0.32	0.03	10.12	0.1	2/13
20:1	14.1	14.3	14.1	13.9	13.7	14.0	13.9	13.7	14.1	14.0	15.1	14.5	14.6	—	11.9[e]	15.0	9.4	14.1	14.4	13.3	13.4	14.04	0.45	3.19	1.3	1/18
20:2n-6	0.1	—	0.4	0.3	0.4	—	0.4	0.4	0.3	0.4	0.1	—	0.4	—	0.3	—	3.2[e]	—	0.3	0.8	0.3	0.30	0.10	32.42	0.3	1/13
20:3n-6	0.2	—	0.4	0.3	0.6	—	0.2	0.3	0.2	0.4	0.4	0.3	0.3	—	—	0.3	1.7[e]	0.3	—	0.9	0.2	0.32	0.11	35.22	0.3	1/14
20:3n-3	0.2	—	0.2	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	—	0.2	—	0.3	1.3[e]	—	0.4	0.1	0.6[e]	0.3	0.23	0.07	29.32	0.2	2/16
20:4n-6	1.2	1.3	1.2	1.3	1.3	1.2	1.3	1.2	1.2	1.3	1.9	1.2	1.2	—	1.2	—	2.1	1.5	1.2	1.7[e]	1.2	1.25	0.09	7.37	0.3	1/17
20:4n-3	1.8	1.9	2.0	1.9	1.9	1.7	1.8	1.8	1.8	1.8	2.3	1.8	1.9	—	1.7	1.7	—	2.0	2.0	2.7[e]	1.7	1.86	0.15	7.86	0.4	1/18
20:5n-3	26.7	27.4	26.5	27.6	25.5	26.3	26.0	25.2	27.1	26.4	22.6	27.2	25.9	—	24.4	26.6	27.5	29.9	27.7	25.3	27.8	26.36	1.54	5.83	4.3	0/18
22:0	—	—	0.1	0.2	—	—	—	0.2	—	0.1	0.1	—	—	—	—	—	0.9	—	—	—	—	0.10	0.07	73.94	0.2	0/07
22:1	10.8	11.4	10.7	10.8	10.4	10.6	10.8	10.2	11.1	9.9	11.0	11.2	10.7	—	9.4	10.5	10.5	7.8[e]	10.0	10.7	10.3	10.59	0.50	4.68	1.4	1/18
22:4n-6	—	—	0.3	0.4	0.5	—	0.2	0.2	1.3	0.3	0.3	—	0.3	—	—	1.3	1.5	—	—	0.4	—	0.48	0.41	84.99	1.1	0/11
22:5n-6	0.3	—	0.3	0.3	0.4	—	0.3	0.3	0.3	0.2	0.3	—	0.3	—	0.7	0.6	0.5	—	0.2	0.3	—	0.33	0.14	43.08	0.4	0/14
22:5n-3	4.4	4.7	4.5	4.3	4.7	4.2	4.3	4.3	4.2	4.2	4.6	4.6	4.5	—	3.7	4.2	—	3.3	4.3	3.8	4.1	4.27	0.36	8.52	1.0	0/18
22:6n-3	20.4	19.8	19.0	19.6	18.5	19.0	19.7	18.3	20.6	17.9	17.5	20.1	18.6	—	16.2	19.6	22.9	15.3	18.3	17.9	20.5	18.68	1.40	7.51	3.9	0/18
24:0	—	—	—	—	—	—	—	0.1	—	—	—	0.3	—	—	—	—	—	—	—	—	—	0.02	0.11	158.70	0.3	1/02
24:1	0.6	—	0.7	—	1.1	0.6	0.7	0.7	0.8	—	0.2	0.7	0.3	—	—	0.8	0.5	—	0.6	—	0.6	0.64	0.23	35.16	0.6	0/12
	mg/g sample																									
20:5n-3	207.0	245.6	218.7	223.8	259.6	211.0	222.9	234.1	225.0	243.4	178.0	220.2	339.1[e]	—	190.3	220.0	262.1	—	239.0	231.1	—	223.11	20.40	9.15	57.1	1/17
22:6n-3	155.0	176.4	153.5	157.0	181.1	149.0	162.6	170.3	168.0	161.8	135.0	159.4	225.8[e]	—	123.5	159.0	213.9	—	155.0	159.8	—	157.90	14.15	8.97	39.6	1/17

[a] Polyglycol column not used. Data are not included in statistical calculations.
[b] Reproducibility standard deviation.
[c] Reproducibility relative standard deviation.
[d] Reproducibility value ($2.8 \times s_R$).
[e] Outlier value, determined by Dixon and/or Grubbs tests, not used in statistical calculations.
[f] Denotes a value reported as "—," "< 0.05," "0," or "tr."

Table 4. Collaborative study results of analysis of Sample 3 (cod liver oil)

	Composition, area %																									
	Laboratory																									
Fatty acids	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17[a]	18	19	20	21[e]	Mean	s_R[b]	RSD_R, %[c]	R[d]	Outliers/ No. labs.
14:0	5.2	5.4	5.6	6.5	4.4	5.2	4.6	5.4	5.2	5.8	5.7	5.8	6.0	5.8	7.5	5.5	4.0	6.7	6.2	5.7	5.3	5.71	0.75	13.12	2.1	0/19
16:0	12.7	12.9	13.5	14.7	10.9	12.9	12.1	13.0	13.2	13.7	12.2	13.8	14.2	13.6	15.3	13.3	9.9	17.8[e]	14.1	12.9	13.3	13.27	1.00	7.51	2.8	1/19
16:1	8.6	7.5	8.6	9.7	7.2	8.4	7.9	9.5	8.6	8.8	8.6	8.8	9.2	9.2	10.2	10.3	11.1	12.5[e]	9.3	8.4	7.8	8.82	0.83	9.39	2.3	1/19
18:0	2.3	2.3	2.4	2.3	2.3	2.3	2.3	2.3	2.3	2.4	2.4	2.4	2.3	2.3	2.1	2.4	1.9	3.3[e]	2.3	2.2	2.3	2.30	0.07	3.05	0.2	1/19
18:1	18.3	17.7	18.4	18.9	17.3	17.8	17.7	18.1	18.4	18.1	17.5	18.4	18.5	18.3	17.7	18.9	12.5	24.3[e]	17.6	17.1	18.1	18.03	0.51	2.82	1.4	1/19
18:2n-6	2.0	2.0	2.1	2.0	2.0	2.0	2.0	2.0	2.0	2.1	2.1	2.1	2.1	2.1	2.0	2.1	8.2[e]	2.6	2.1	2.0	2.0	2.04	0.05	2.37	0.1	1/19
18:3n-3	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	0.9	1.0	0.1[e]	1.0	1.0	1.0	1.0	1.0	1.3	1.1	1.0	1.0	1.1	1.01	0.04	3.86	0.1	1/19
18:4n-3	2.0	1.9	2.0	1.9	2.0	2.0	1.9	1.9	2.0	2.0	2.0	1.9	2.0	1.9	1.9	2.0	4.3	2.1	2.0	2.2	1.9	1.97	0.08	4.24	0.2	0/19
20:0	0.1	—[f]	0.1	0.1	—	—	0.1	0.1	0.2	0.2	1.0	0.1	0.3	0.2	—	—	4.3[e]	0.3	0.1	4.4[e]	0.1	0.15	0.08	53.89	0.2	2/15
20:1	10.7	9.4	10.1	9.5	10.9	10.1	10.3	9.7	10.2	9.4	10.4	10.3	9.9	9.9	8.0	10.1	1.2[e]	10.3	9.5	4.4[e]	10.3	10.04	0.45	4.46	1.3	2/19
20:2n-6	—	—	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	—	—	0.4	0.3	0.2	0.5	0.3	0.31	0.08	25.77	0.2	0/15
20:3n-6	—	—	0.1	0.1	0.2	—	—	0.1	—	0.1	0.1	—	—	0.1	—	—	0.5	0.2	—	0.1	0.1	0.09	0.07	67.73	0.2	0/12
20:3n-3	0.1	—	0.1	0.1	—	0.2	0.2	0.2	—	0.1	0.1	—	0.1	0.1	0.1	0.5[e]	0.3	0.3	0.1	0.2	0.1	0.14	0.06	40.34	0.2	1/15
20:4n-6	0.4	—	0.5	0.4	0.8	0.5	0.5	0.5	0.4	0.5	—	0.5	0.4	0.4	0.4	—	0.6	0.4	0.4	0.6	0.4	0.42	0.19	43.83	0.5	0/18
20:4n-3	0.6	—	0.6	0.6	0.8	0.7	0.6	0.6	0.5	0.6	0.7	0.6	0.6	0.6	0.5	0.6	—	0.4	0.6	1.0[e]	0.8	0.59	0.08	13.76	0.2	1/18
20:5n-3	8.1	7.8	7.9	7.6	8.4	7.9	8.2	7.4	8.1	7.1	8.0	7.7	7.8	7.6	6.8	8.4	7.4	5.3[e]	7.5	7.4	7.9	7.76	0.43	5.48	1.2	1/19
22:0	1.2[e]	—	0.2	0.3	—	—	0.4	0.4	0.3	0.3	—	0.3	—	0.3	—	0.3	1.4[e]	—	0.3	—	—	0.32	0.03	11.77	0.1	2/12
22:1	8.8	7.7	9.4	9.1	10.2	9.3	9.9	8.8	9.5	8.6	9.9	9.4	9.3	9.4	7.2	8.8	7.2	7.6	8.4	9.1	8.8	8.96	0.80	8.88	2.2	0/19
22:4n-6	—	—	0.1	0.5	0.3	0.3	0.2	0.2	—	0.1	—	—	—	0.1	—	0.3	0.5	—	0.1	0.3	—	0.21	0.16	67.99	0.4	0/12
22:5n-6	—	—	0.1	0.2	0.2	0.1	0.2	0.1	—	0.1	0.1	—	—	0.1	—	—	—	—	0.1	0.1	—	0.13	0.04	31.75	0.1	0/11
22:5n-3	1.1	—	1.0	1.0	1.2	1.0	1.1	1.0	1.0	0.9	1.1	1.0	0.9	1.0	0.8	1.1	—	0.5[e]	0.9	1.1	1.0	1.01	0.10	9.73	0.3	1/18
22:6n-3	7.7	6.9	6.9	7.2	8.5	7.0	7.4	6.4	7.4	6.5	7.5	6.9	6.8	6.5	5.4	7.2	7.6	3.9[e]	6.0	7.4	7.4	6.97	0.69	9.86	1.9	1/19
24:0	—	—	—	—	—	—	0.3	0.3	0.3	0.4	0.4	—	—	0.1	—	—	0.5	—	—	—	0.9	0.24	0.14	59.08	0.4	0/07
24:1	2.6	—	0.3	—	0.8	0.4	0.8	0.7	0.7	0.5	1.4	1.2	—	0.6	0.4	2.5	1.1	—	0.6	0.5	0.6	0.93	0.73	78.73	2.0	0/15
	mg/g sample																									
20:5n-3	57.0	61.2	65.2	66.0	63.7	63.4	63.3	65.1	62.0	66.9	70.0	118.7[e]	54.8	61.1	76.9	62.0	54.3	—	68.5	65.0	—	64.27	4.98	7.72	13.9	1/18
22:6n-3	53.0	53.2	56.3	61.9	62.4	54.6	56.6	56.2	55.0	60.1	63.0	104.4[e]	47.1	51.0	59.2	52.0	54.2	—	53.4	63.3	—	56.37	4.68	8.30	13.1	1/18

[a] Polyglycol column not used. Data are not included in statistical calculations.
[b] Reproducibility standard deviation.
[c] Reproducibility relative standard deviation.
[d] Reproducibility value ($2.8 \times s_R$).
[e] Outlier value, determined by Dixon and/or Grubbs tests, not used in statistical calculations.
[f] Denotes a value reported as "—," "< 0.05," "0," or "tr."

Table 5. Collaborative study results of analysis of Sample 4 (MaxEPA®)

	Composition, area %																									
	Laboratory																									
Fatty acids	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17[e]	18	19	20	21[a]	Mean	s_R[b]	RSD_R, %[c]	R[d]	Outliers/ No. labs.
14:0	6.5	7.3	7.4	7.7	5.6	6.8	6.2	7.0	6.7	7.5	6.6	6.8	7.6	6.8	10.1[e]	6.9	5.1	6.9	8.0	7.1	6.8	6.97	0.58	8.31	6.6	1/19
16:0	14.2	15.7	16.1	16.1	12.9	14.9	14.3	15.2	15.1	16.3	12.6	15.0	15.6	14.7	15.8	15.0	12.0	15.4	16.4	14.6	15.3	15.04	1.03	0.85	2.9	0/19
16:1	7.6	8.2	8.3	8.5	6.7	7.8	7.6	8.8	7.7	8.4	7.9	7.7	8.2	8.2	8.3	11.2[e]	9.2	9.1	8.5	7.6	7.2	8.06	0.55	6.80	0.5	1/19
18:0	3.0	3.2	3.1	2.8	3.1	3.0	1.8	3.0	1.8	3.2	3.4	3.1	2.9	3.0	2.7	3.1	2.6	3.2	3.0	2.8	3.0	2.90	0.42	14.43	1.2	0/19
18:1	13.8	14.5	14.6	14.1	13.8	13.9	14.1	14.1	14.1	14.3	13.9	14.0	14.1	14.2	13.3	14.4	10.4	14.8	14.1	13.0	13.9	14.06	0.42	2.98	1.2	0/19
18:2n-6	1.1	1.2	1.2	1.1	1.1	1.2	1.2	1.2	1.1	1.2	1.3	1.1	1.2	1.2	1.9	1.2	5.5[e]	1.2	1.2	1.1	1.2	1.16	0.05	4.65	0.2	1/19
18:3n-3	0.7	0.7	0.7	0.6	0.7	0.7	0.7	0.7	0.6	0.7	0.5	0.7	0.7	0.7	0.6	0.7	1.2	0.8	0.6	0.7	0.7	0.66	0.06	9.16	0.2	0/19
18:4n-3	2.1	2.1	2.2	2.0	2.0	2.1	2.1	2.0	2.0	2.1	2.3	1.9	2.1	2.0	1.8	2.1	3.3	2.4	2.1	2.0	2.0	2.07	0.13	6.30	0.4	0/19
20:0	0.4	—[f]	0.4	0.4	—	—	0.4	0.4	0.4	0.4	0.8	0.4	0.3	0.4	—	0.4	2.7	0.5	0.3	1.0	0.4	0.47	0.17	37.24	0.5	0/15
20:1	3.3	3.1	3.1	2.9	4.2	3.2	3.3	3.1	3.0	3.1	3.7	3.3	3.1	3.2	0.5[e]	3.5	1.2	3.5	3.0	1.9[e]	3.1	3.25	0.32	9.70	0.9	2/19
20:2n-6	0.2	—	0.2	0.2	0.2	0.2	0.2	0.2	—	0.2	0.2	0.2	0.2	0.1	0.1	—	0.5	0.2	0.1	0.2	0.2	0.16	0.03	21.35	0.1	0/16
20:3n-6	0.1	—	0.1	0.1	0.3	—	0.1	0.1	—	0.1	0.1	0.1	0.1	0.1	—	—	1.5	0.2	0.1	0.2	0.1	0.13	0.07	50.84	0.2	0/15
20:3n-3	0.1	—	0.1	0.1	0.2	—	0.1	0.3	—	0.1	0.1	—	0.1	0.1	0.1	0.9[e]	—	0.3	0.1	0.1	0.1	0.15	0.07	47.93	0.2	1/15
20:4n-6	0.8	0.9	0.8	0.8	1.0	0.8	0.9	0.9	0.8	0.8	1.0	0.8	0.8	0.8	0.7	—	0.4[e]	0.9	0.8	0.8	0.8	0.83	0.07	8.86	0.2	1/19
20:4n-3	0.8	0.8	0.8	0.8	0.9	0.9	0.8	0.8	0.8	0.8	1.0	0.8	0.7	0.8	0.6	0.8	—	0.9	0.7	0.7	0.8	0.80	0.08	10.44	0.2	0/19
20:5n-3	17.9	17.8	17.2	16.9	18.4	17.4	17.5	16.3	18.1	15.6	15.8	16.8	17.3	16.8	14.4	17.5	16.4	19.9	16.2	15.2	17.4	16.99	1.26	7.42	3.5	0/19
22:0	—	—	—	0.2	—	—	0.1	0.1	—	0.1	0.1	0.1	0.1	0.1	—	—	1.2[e]	—	0.1	—	0.1	0.11	0.02	15.93	0.1	1/10
22:1	2.5	2.5	2.4	2.4	2.8	2.5	2.7	2.4	2.5	2.3	3.0	2.8	2.3	2.3	2.0	2.1	3.1	2.4	2.3	2.2	2.6	2.44	0.24	10.02	0.7	0/19
22:4n-6	—	—	0.3	0.2	0.3	—	0.1	0.1	0.1	0.3	0.1	0.1	0.3	0.1	—	—	0.8	—	0.1	0.2	0.1	0.16	0.09	56.22	0.3	0/14
22:5n-6	0.3	—	—	0.3	0.4	0.3	0.3	0.3	0.3	0.1[e]	0.3	0.3	—	0.3	—	0.6[e]	—	—	0.2	1.4[e]	—	0.29	0.04	13.14	0.1	3/14
22:5n-3	2.3	2.2	2.0	1.8	2.3	2.1	2.2	2.0	2.2	1.8	2.5	2.2	1.9	2.0	1.5	2.1	—	2.1	1.8	2.5	2.1	2.07	0.25	12.13	0.7	0/19
22:6n-3	13.5	12.6	11.9	12.1	13.4	12.3	12.5	11.2	13.3	10.6	12.4	12.4	12.0	11.4	8.8	12.6	12.3	15.1	10.5	14.5	12.8	12.27	1.44	11.74	4.0	0/19
24:0	—	—	—	—	—	—	0.1	—	0.1	0.1	—	0.1	—	0.1	—	—	—	—	0.1	—	0.1	0.06	0.07	70.06	0.2	0/07
24:1	0.6	—	—	—	0.4	—	0.6	0.5	0.6	—	—	0.7	—	0.5	—	0.5	0.7	—	0.5	—	0.6	0.44	0.23	52.96	0.7	0/11
	mg/g sample																									
20:5n-3	135.0	144.4	148.8	158.6	125.7	141.0	143.8	153.6	137.0	152.2	144.0	244.2	154.3	145.8	232.6	170.0	131.7	—	156.0	147.2	—	157.46	31.10	9.75	87.1	0/18
22:6n-3	100.0	100.0	101.0	112.4	88.0	98.2	101.4	105.2	99.0	101.9	104.0	176.2[e]	104.8	97.0	138.9	102.0	97.1	—	99.0	137.3	—	105.30	13.27	2.60	37.1	1/18

[e] Polyglycol column not used. Data are not included in statistical calculations.
[b] Reproducibility standard deviation.
[c] Reproducibility relative standard deviation.
[d] Reproducibility value ($2.8 \times s_R$).
[a] Outlier value, determined by Dixon and/or Grubbs tests, not used in statistical calculations.
[f] Denotes a value reported as "—," "< 0.05," "0," or "tr."

Table 6. Collaborative study results of analysis of Sample 5 (Promega®)

Fatty acids	Composition, area %: Laboratory 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17[a]	18	19	20	21[a]	Mean	s_R[b]	RSD_R, %[c]	R[d]	Outliers/ No. labs.
14:0	4.9	5.5	5.5	5.8	4.3	4.7	4.7	5.3	4.7	5.6	5.5	5.6	5.7	5.1	7.2	5.3	4.8	10.8[e]	5.9	5.5	5.1	5.37	0.64	11.98	1.8	1/19
16:0	7.2	8.0	8.2	8.0	6.7	7.4	7.5	7.8	7.1	8.4	7.9	8.2	8.2	7.6	7.9	7.8	6.0	13.7[e]	8.2	7.9	7.8	7.77	0.44	5.66	1.2	1/19
16:1	8.4	8.6	9.3	9.5	7.5	8.2	8.5	9.9	7.6	9.5	8.9	9.1	9.3	8.4	9.1	17.0	9.6	16.6	9.4	9.0	8.3	9.67	2.59	26.73	7.2	0/19
18:0	0.8	4.2[e]	0.8	0.8	0.8	0.8	0.9	0.8	0.7	0.9	0.9	0.9	0.8	0.8	0.8	0.9	0.8	7.3[e]	0.8	0.9	0.8	0.82	0.05	5.56	0.1	2/19
18:1	9.6	10.3	10.3	9.8	10.8	9.8	9.9	10.1	9.2	10.3	10.4	10.3	10.0	9.9	9.3	10.3	9.2	11.5	10.0	9.8	9.8	10.17	0.68	6.71	1.9	0/19
18:2n-6	1.1	1.2	1.2	1.1	1.2	1.1	1.2	1.2	1.0	1.2	1.3	1.2	1.1	1.1	1.1	1.2	3.2	1.3	1.2	1.1	1.2	1.16	0.07	5.97	0.2	0/19
18:3n-3	0.9	1.0	1.0	0.9	0.9	1.0	1.0	1.0	0.8	1.0	0.2	1.0	0.9	0.9	0.8	1.0	6.6[e]	1.0	1.0	0.9	1.0	0.94	0.06	5.98	0.2	1/19
18:4n-3	4.4	4.6	0.2[e]	4.3	4.3	4.4	4.5	4.3	4.0	4.5	4.8	4.3	4.5	4.4	4.0	4.5	0.4	5.0	4.5	4.3	4.4	4.42	0.24	5.45	0.7	1/19
20:0	0.1	—[f]	0.1	0.5	—	—	0.1	0.1	—	0.2	1.1	0.1	0.2	0.2	—	—	1.7[e]	0.2	0.1	0.6	0.1	0.19	0.16	84.25	0.4	1/14
20:1	1.5	1.4	1.5	1.4	1.2	1.5	1.5	1.4	1.2	1.5	1.6	1.5	1.3	1.5	1.2	0.7	0.3	1.0	1.4	0.9	1.5	1.32	0.24	18.13	0.7	0/19
20:2n-6	0.1	—	0.1	0.1	0.1	0.1	0.1	0.1	—	0.1	0.1	—	—	0.1	—	—	—	—	0.1	0.1	0.1	0.10	0.01	10.29	0.1	0/14
20:3n-6	0.1	—	0.1	0.2	0.4[e]	—	0.2	0.2	—	0.1	0.1	—	0.1	0.1	—	0.2	0.3	0.1	0.1	0.3	0.2	0.14	0.05	34.18	0.1	1/14
20:3n-3	—	—	0.1	0.2	0.2	0.2	0.1	0.1	—	0.1	0.1	—	—	0.1	—	0.8[e]	0.9[e]	0.2	0.1	0.2	0.1	0.14	0.04	27.47	0.1	2/14
20:4n-6	0.8	1.2[e]	0.8	0.7	0.8	0.8	0.8	0.8	0.9	0.8	0.9	0.8	0.7	0.7	0.6	—	—	0.6	0.7	0.7	0.8	0.75	0.08	10.85	0.2	2/19
20:4n-3	1.1	0.8	1.1	1.0	1.2	1.2	1.2	1.1	1.0	1.2	1.4[e]	1.2	1.1	1.1	0.9	1.2	—	0.8	1.2	1.1	1.2	1.09	0.15	13.77	0.4	1/19
20:5n-3	29.3	29.4	28.7	27.8	29.9	28.8	28.6	27.1	27.8	26.8	24.1	28.0	28.7	28.4	24.9	29.1	27.0	21.6	27.3	26.8	29.1	27.54	2.06	7.49	5.8	0/19
22:0	—	—	—	0.1	—	—	—	—	—	—	1.1	—	—	0.1	—	—	1.4[e]	—	—	—	—	0.03	0.38	141.42	1.1	1/06
22:1	0.9	0.9	1.0	1.1	1.2	1.1	1.1	1.1	0.8	1.0	1.2	0.9	0.9	1.0	1.3	0.9	1.7	—	0.8	1.1	1.1	1.02	0.14	13.35	0.04	0/18
22:4n-6	—	—	—	0.1	0.1	—	—	0.1	0.4	0.2	—	—	—	0.1	—	0.9[e]	0.9	—	0.1	—	—	0.13	0.14	93.55	0.4	1/09
22:5n-6	0.2	—	—	0.2	0.2	0.2	0.2	0.3	0.5	0.1[e]	0.2	—	0.2	0.2	0.2	—	—	—	0.1	1.2[e]	—	0.20	0.07	28.69	0.2	2/14
22:5n-3	2.4	2.3	2.2	2.0	2.4	2.3	2.3	2.1	2.1	2.0	2.5	2.3	2.1	2.2	1.7	2.3	—	1.2[e]	2.0	2.0	2.3	2.18	0.19	8.84	0.5	1/19
22:6n-3	14.1	13.0	12.3	12.2	13.5	13.1	13.0	12.0	12.6	11.2	12.9	12.7	12.5	12.5	10.1	13.3	13.3	7.1[e]	11.7	11.4	13.8	12.45	0.93	7.50	2.6	1/19
24:0	—	—	—	—	—	—	0.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.00	0.02	100.00	0.1	1/05
24:1	0.2	—	—	—	0.3	—	0.6	0.2	0.2	—	—	0.2	—	0.1	—	—	0.4	—	—	—	0.2	0.17	0.07	102.20	0.2	0/10
mg/g sample																										
20:5n-3	232.0	240.4	253.7	262.9	245.8	235.0	242.7	250.8	234.0	262.4	213.0	412.9[e]	236.6	239.4	228.4	240.6	214.6	—	256.0	230.0	—	241.40	12.98	5.38	36.3	1/18
22:6n-3	109.0	106.4	106.7	114.8	106.5	105.0	109.1	110.9	104.0	107.5	107.0	183.4[e]	100.6	102.8	91.0	108.0	103.5	—	107.0	96.3	—	105.44	5.52	5.23	15.5	1/18

[a] Polyglycol column not used. Data are not included in statistical calculations.
[b] Reproducibility standard deviation.
[c] Reproducibility relative standard deviation.
[d] Reproducibility value ($2.8 \times s_R$).
[e] Outlier value, determined by Dixon and/or Grubbs tests, not used in statistical calculations.
[f] Denotes a value reported as "—," "<0.05," "0," or "tr."

Table 7. Collaborative study results of analysis of Sample 6 (blind duplicate, steam-deodorized menhaden oil)

	Composition, area %																									
	Laboratory																									
Fatty acids	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17[a]	18	19	20	21[a]	Mean	s_R[b]	RSD_R, %[c]	R[d]	Outliers/ No. labs.
14:0	8.1	7.1	8.1	10.9	6.7	7.9	7.4	8.0	7.6	8.3	7.7	8.8	8.7	8.8	13.4[e]	8.2	6.0	9.6	9.3	8.2	7.8	8.30	0.96	11.62	2.7	1/19
16:0	18.4	17.9	19.3	21.8	16.5	18.6	18.0	18.5	19.7	19.6	15.2	19.9	19.3	19.2	18.9	19.1	15.3	24.5	19.9	17.8	19.1	19.05	1.91	10.02	5.3	0/19
16:1	11.7	10.9	11.7	13.6	10.0	11.3	11.3	12.4	11.3	11.9	10.8	11.4	11.9	11.7	11.9	13.8	12.9	15.6	12.4	10.9	10.9	11.91	1.27	10.66	3.5	0/19
18:0	3.1	3.2	3.2	2.7	3.1	3.7	3.1	3.0	3.1	3.2	3.5	3.2	3.0	3.0	2.3[e]	3.3	2.6	3.8[e]	3.0	2.8	3.1	3.09	0.18	5.71	0.5	2/19
18:1	11.9	12.2	12.3	11.2	11.9	11.7	11.9	11.8	12.1	12.0	12.4	12.3	11.9	11.7	9.7[e]	12.5	12.2	14.6[e]	11.5	10.8	11.9	11.88	0.43	3.62	1.2	2/19
18:2n-6	1.2	1.2	1.4	1.1	1.2	1.2	1.2	1.2	1.1	1.2	1.3	1.2	1.2	1.2	0.9	1.2	2.3	1.5	1.1	1.1	1.1	1.20	0.13	10.69	0.4	0/19
18:3n-3	0.8	0.8	0.7	0.7	0.8	0.8	0.8	0.8	0.7	0.8	0.3	0.8	0.4	0.7	0.6	0.8	6.0	1.0	0.8	1.0	0.8	0.75	0.17	23.14	0.5	0/19
18:4n-3	3.0	3.0	3.0	2.7	2.9	2.9	2.9	2.9	2.9	2.9	3.3	2.8	2.9	2.8	2.4	3.1	0.5[e]	3.5[e]	2.9	2.9	2.8	2.92	0.13	4.48	0.4	2/19
20:0	0.2	—[f]	0.2	0.2	—	—	0.2	0.2	0.2	0.2	0.9[e]	0.2	0.2	0.2	—	0.2	2.3[e]	0.3	0.2	—	0.2	0.21	0.02	8.52	0.1	2/14
20:1	1.7	1.8	2.0	1.4	2.5	1.9	1.9	1.9	1.9	1.6	2.1	1.6	1.7	1.8	1.2	2.0	0.5	1.5	1.5	2.2	1.9	1.80	0.29	16.24	0.8	0/19
20:2n-6	0.2	—	0.2	0.1	0.2	0.2	0.2	0.2	—	0.2	0.1	0.1	0.1	0.1	—	—	0.4	—	0.1	0.2	0.2	0.15	0.04	25.41	0.1	0/14
20:3n-6	0.2	—	0.2	0.3[e]	0.4[e]	0.2	0.2	0.2	—	0.2	—	0.1	0.2	0.2	—	0.2	0.4	—	0.2	0.2	0.2	0.19	0.03	16.08	0.1	2/15
20:3n-3	—	—	0.1	0.2	0.3	0.2	0.2	0.2	—	0.1	0.2	—	0.1	0.1	—	0.9[e]	1.0[e]	0.2	0.1	0.4	0.1	0.16	0.05	28.55	0.1	2/14
20:4n-6	0.8	1.0	0.3[e]	0.7	1.0	0.9	0.9	0.8	0.8	0.8	1.0	0.9	0.8	0.8	0.6	—	0.6	0.9	0.8	1.0	0.8	0.84	0.10	12.08	0.3	2/19
20:4n-3	1.3	1.5	1.3	1.1	1.4	1.3	1.3	1.3	1.3	1.3	1.5	1.3	1.2	1.2	0.8[e]	1.3	—	1.3	1.2	2.1[e]	1.4	1.29	0.11	8.17	0.3	2/19
20:5n-3	14.6	15.3	14.0	11.4	14.9	13.6	13.9	12.9	14.8	12.4	13.7	13.5	13.7	13.2	9.7	14.2	14.0	14.0	12.7	12.6	14.1	13.42	1.31	9.78	3.7	0/19
22:0	—	—	0.3	0.2	—	—	0.2	0.2	—	0.1	0.1	—	0.1	0.2	—	—	0.6	—	0.2	—	0.1	0.17	0.08	48.00	0.2	0/10
22:1	0.9	1.6	1.5	1.2	1.6	1.3	1.4	1.3	1.1	1.4	1.7	1.2	1.6	1.4	1.2	1.3	1.2	—	1.1	3.0[e]	0.9	1.35	0.22	16.05	0.6	1/18
22:4n-6	—	—	0.1	0.3	0.3	0.2	0.2	0.3	—	0.2	0.1	—	0.2	0.2	—	0.7	0.9[e]	—	0.1	0.2	0.1	0.20	0.08	38.29	0.2	1/13
22:5n-6	0.2	—	0.1	0.2	0.2	0.2	0.2	0.2	—	0.1	0.2	—	—	0.1	0.1	—	—	—	0.1	0.3	—	0.16	0.08	50.60	0.2	0/14
22:5n-3	2.3	2.4	2.0	1.5	2.3	2.1	2.1	1.9	2.2	1.8	2.3	2.1	1.9	1.9	1.2	2.2	—	1.5	1.8	1.9	2.2	1.97	0.32	16.20	0.9	0/19
22:6n-3	9.3	9.5	8.1	6.2	9.1	7.9	8.2	7.5	9.1	7.1	8.7	8.0	7.9	7.6	4.8	8.6	9.4	6.2	6.7	7.5	8.7	7.77	1.20	15.48	3.4	0\19
24:0	—	—	—	—	—	—	0.1	0.1	0.3	0.1	0.1	—	—	0.1	0.2	—	—	—	—	—	0.3	0.14	0.07	48.38	0.2	0/06
24:1	—	—	0.1	—	0.4	0.2	0.4	0.4	0.4	0.2	0.5	0.6	—	0.4	—	0.6	0.6	—	—	0.4	0.3	0.36	0.14	41.22	0.4	0/13
	mg/g sample																									
20:5n-3	124.0	108.8	120.2	133.1	135.8	116.0	116.5	118.2	107.0	119.7	118.0	230.6[e]	103.2	102.7	121.9	112.0	105.0	—	126.0	118.3	—	116.54	9.25	7.86	25.9	1/18
22:6n-3	77.0	67.6	67.6	71.3	79.6	65.5	67.8	68.3	65.0	67.5	70.0	134.2[e]	57.8	57.7	59.5	66.0	69.0	—	65.5	69.2	—	67.23	5.76	8.57	16.1	1/18

[a] Polyglycol column not used. Data are not included in statistical calculations.
[b] Reproducibility standard deviation.
[c] Reproducibility relative standard deviation.
[d] Reproducibility value ($2.8 \times s_R$).
[e] Outlier value, determined by Dixon and/or Grubbs tests, not used in statistical calculations.
[f] Denotes a value reported as "—," "< 0.05," "0," or "tr."

Table 8. Statistical evaluation of collaborative study of a marine oil and blind duplicate on capillary GC column coated with a bonded polyglycol liquid phase[a]

Fatty acid	Mean	s_R	RSD_R, %	R	s_r	RSD_r, %	r	Outliers/ No. laboratories
	Composition, area %							
14:0	8.27	0.95	11.26	2.7	0.49	5.79	1.4	1/19
16:0	18.72	1.36	7.27	3.9	0.54	2.89	1.5	1/19
16:1	11.99	1.47	12.26	4.2	0.51	4.28	1.5	0/19
18:0	3.10	0.15	4.76	0.4	0.06	1.84	0.2	2/19
18:1	11.90	0.34	2.88	1.0	0.19	1.57	0.5	2/19
18:2n-6	1.19	0.06	4.65	0.2	0.01	1.19	0.0	4/19
18:3n-3	0.75	0.17	22.06	0.5	0.04	5.58	0.1	0/19
18:4n-3	2.91	0.08	2.85	0.2	0.04	1.52	0.1	3/19
20:0	0.21	0.02	8.07	0.1	0.01	5.46	0.0	2/14
20:1	1.81	0.27	14.98	0.8	0.13	7.27	0.4	0/19
20:2n-6	0.15	0.04	25.87	0.1	0.01	8.64	0.0	1/14
20:3n-6	0.19	0.04	21.53	0.1	0.03	17.35	0.1	2/16
20:3n-3	0.16	0.06	30.63	0.2	0.01	3.11	0.0	3/15
20:4n-6	0.84	0.09	11.62	0.3	0.02	2.53	0.1	3/19
20:4n-3	1.30	0.10	7.74	0.3	0.04	3.46	0.1	3/19
20:5n-3	13.80	0.80	5.93	2.3	0.25	1.85	0.7	2/19
22:0	0.18	0.11	59.75	0.3	0.02	8.92	0.0	0/12
22:1	1.37	0.23	16.27	0.6	0.11	7.86	0.3	2/18
22:4n-6	0.19	0.11	49.94	0.3	0.06	26.34	0.2	1/14
22:5n-6	0.16	0.08	48.89	0.2	0.03	18.61	0.1	0/15
22:5n-3	1.97	0.37	18.99	1.1	0.13	6.72	0.4	0/19
22:6n-3	7.87	1.23	15.74	3.5	0.29	3.72	0.8	1/19
24:0	0.12	0.07	61.03	0.2	0.01	10.93	0.0	0/07
24:1	0.32	0.15	44.16	0.4	0.03	7.37	0.1	2/16
	mg/g sample							
20:5n-3	116.54	8.56	7.03	24.2	7.17	5.89	20.3	1/18
22:6n-3	66.61	4.74	6.80	13.4	3.68	5.27	10.4	1/18

[a] s_R = reproducibility standard deviation, RSD_R = reproducibility relative standard deviation; R = reproducibility value ($2.8 \times s_R$); s_r = repeatability standard deviation; RSD_r = repeatability relative standard deviation; r = repeatability value ($2.8 \times s_r$).

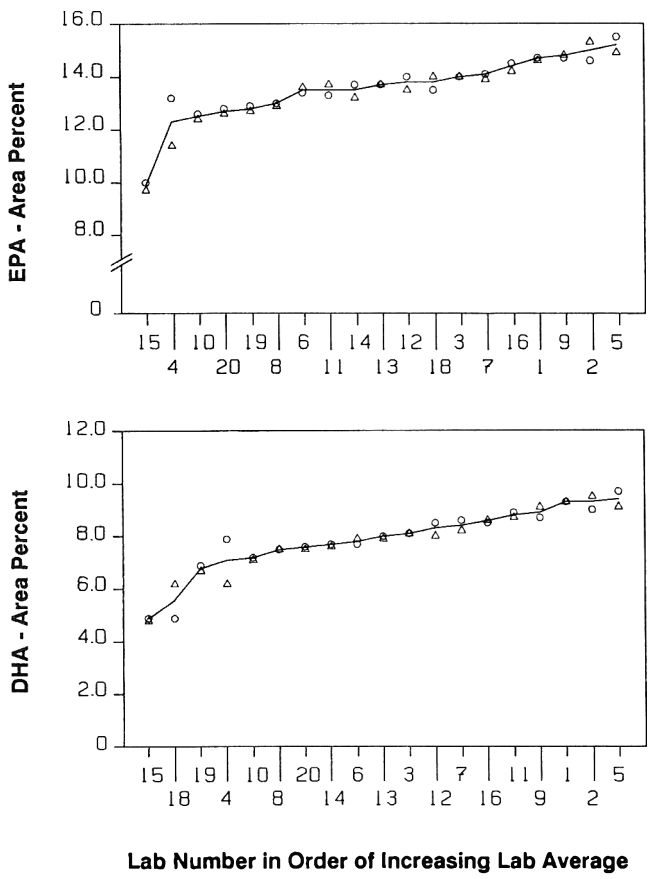

Figure 1. GC analysis of EPA and DHA in Samples 1 (o) and 6 (Δ) (blind duplicate): concentration in area percent vs collaborator number in ascending order.

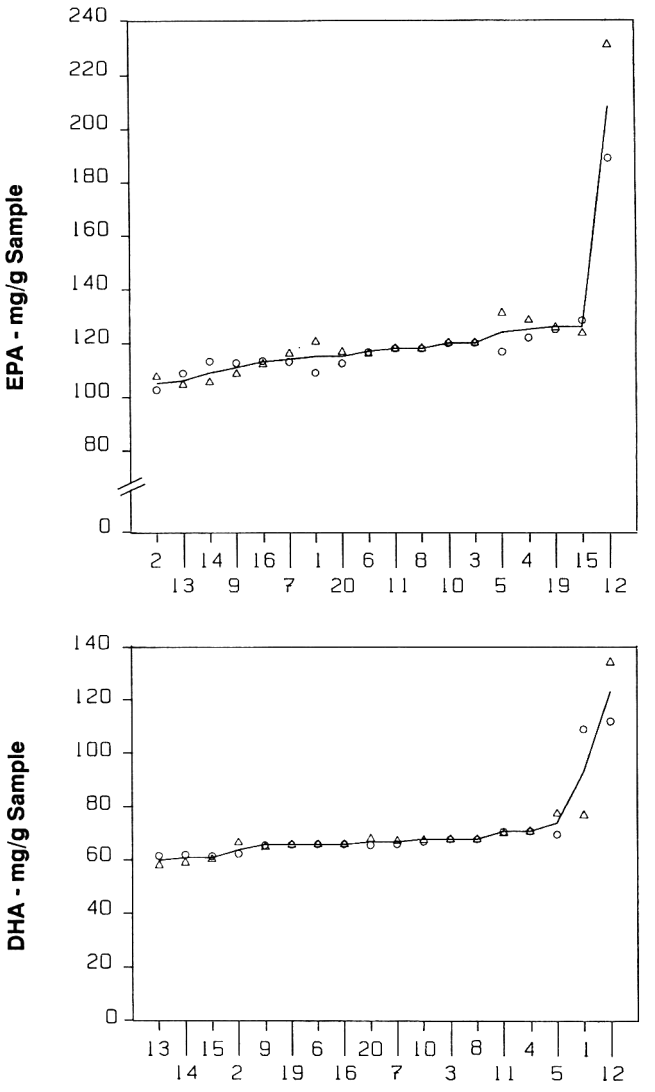

Figure 2. GC analysis of EPA and DHA in Samples 1 (o) and 6 (Δ) (blind duplicate): concentration in mg/g sample vs collaborator number in ascending order.

orator responded that the internal standard had been improperly prepared and requested that the data be disregarded. Collaborator 12 originally reported weights of EPA and DHA ranging from 2800 to 10 000 mg/g in the 6 samples, clearly reflecting errors in the calculations. Subsequently, recalculated values were submitted and are listed in Tables 2–7.

The results of between-laboratory reproducibility (RSD_R) and within-laboratory repeatability (RSD_r) calculations derived from the analysis of Sample 1 and its blind duplicate, Sample 6, are given in Table 8. The ranges and, hence, the variabilities in the determination of the area percentages of EPA and DHA are typified in Figure 1 and those for the absolute weights in Figure 2.

All calculations except the reproducibility values (R) and repeatability values (r) were performed by using the computer program FDACHEMIST, which was developed for the statistical analysis of collaborative study data (2). The identities (but not the existence) of Dixon and/or Grubbs between-laboratory outlier values were determined by visual inspection, referred to by Albert as the "ultimate outlier test" (3), and are indicated in Tables 2–7. The Cochran test was used to calculate within-laboratory outlier values, also by using FDACHEMIST.

A summary of the statistical performance of the method for all of the 26 analytes in the 5 oils and the ethyl ester concentrate is given in the method as Method Performance. Because of the large amount of data generated in the analysis of the oils, ranges for s_R and RSD_R, rather than individual values, are listed for this matrix. Repeatability standard deviations (s_r and RSD_r) could be calculated only for the analysis of one oil and its blind duplicate.

Collaborators' Comments

Collaborator 6 noted that a labeled chromatogram of fish oil ethyl esters would have aided in identification of the components in Sample 2.

Using a DB-Wax column, Collaborator 11 observed coelution of some sample components with the internal standard; correction was made for this coelution. This collaborator also commented that when their chromatographic system is "functioning properly" (phrase not defined), it gives response factors that are within ±1% of the theoretical response factors published by Craske and Bannon (4). When it is not functioning

Table 9. Comparison of 1977 AOCS Smalley, 1979 AOAC, and 1988 AOAC collaborative study results

Fatty Acid	14:0	16:0	16:1	18:0	18:1	18:2	20:1	20:5	22:1	22:6
					1977 AOCS					
N	55	53	55	55	55	51	55	41	52	33
Mean, %	4.2	8.6	13.1	1.2	13.7	1.2	18.8	6.6	21.7	3.2
SD	0.60	0.60	1.28	0.51	0.67	0.63	1.25	0.66	2.87	0.42
CV, %	14.3	6.9	9.8	28.2	4.9	53.4	6.6	10.0	13.2	13.1
					1979 AOAC					
N	15	14	14	15	15	15	14	13	13	14
Mean, %	4.1	8.7	13.2	1.9	13.9	0.9	18.7	7.0	21.5	3.5
SD	0.44	0.69	0.97	0.39	0.77	0.41	0.72	0.59	1.29	0.69
CV, %	10.7	7.9	7.4	20.6	5.6	45.1	3.9	8.5	6.0	19.9
					1988 AOAC					
Sample 1										
N	19	18	19	17	17	18	17	18	17	19
Mean, %	8.3	18.7	12.1	3.1	11.9	1.2	1.8	13.8	1.4	7.9
SD	0.98	1.31	1.65	0.11	0.23	0.07	0.15	0.79	0.25	1.27
CV, %	11.7	7.0	13.6	3.6	1.9	5.6	8.0	5.8	17.2	16.1
Sample 2										
N	17	17	15	17	17	17	17	18	17	18
Mean, %	0.4	0.9	0.3	2.1	8.7	0.8	14.2	26.4	10.6	18.7
SD	0.06	0.11	0.10	0.15	0.50	0.06	0.45	1.54	0.50	1.40
CV, %	17.1	12.2	31.0	7.0	5.7	7.9	3.2	5.8	4.7	7.5
Sample 3										
N	19	18	18	18	18	18	17	18	19	18
Mean, %	5.7	13.3	8.8	2.3	18.0	2.0	10.0	7.8	9.0	7.0
SD	0.75	1.00	0.83	0.07	0.51	0.05	0.45	0.43	0.80	0.69
CV, %	13.1	7.5	9.4	3.1	2.8	2.4	4.5	5.5	8.9	9.9
Sample 4										
N	18	19	18	19	19	18	17	19	19	19
Mean, %	7.0	15.0	8.1	2.9	14.1	1.2	3.3	17.0	2.4	12.3
SD	0.58	1.03	0.55	0.42	0.42	0.05	0.32	1.26	0.24	1.44
CV, %	8.3	6.9	6.9	14.4	3.0	4.7	9.7	7.4	10.1	11.7
Sample 5										
N	17	18	19	17	19	18	19	19	18	18
Mean, %	5.4	7.8	9.7	0.8	10.1	1.2	1.3	27.5	1.0	12.5
SD	0.64	0.44	2.59	0.05	0.52	0.07	0.24	2.06	0.14	0.93
CV, %	12.0	5.7	26.7	5.6	5.2	6.0	18.1	7.5	13.4	7.5
Sample 6										
N	18	19	19	17	17	19	19	19	18	19
Mean, %	8.3	19.1	11.9	3.1	11.9	1.2	1.8	13.4	1.4	7.8
SD	0.96	1.91	1.27	0.18	0.42	0.13	0.29	1.31	0.22	1.20
CV, %	11.6	10.0	10.6	5.7	3.6	10.7	16.2	9.8	16.1	15.5

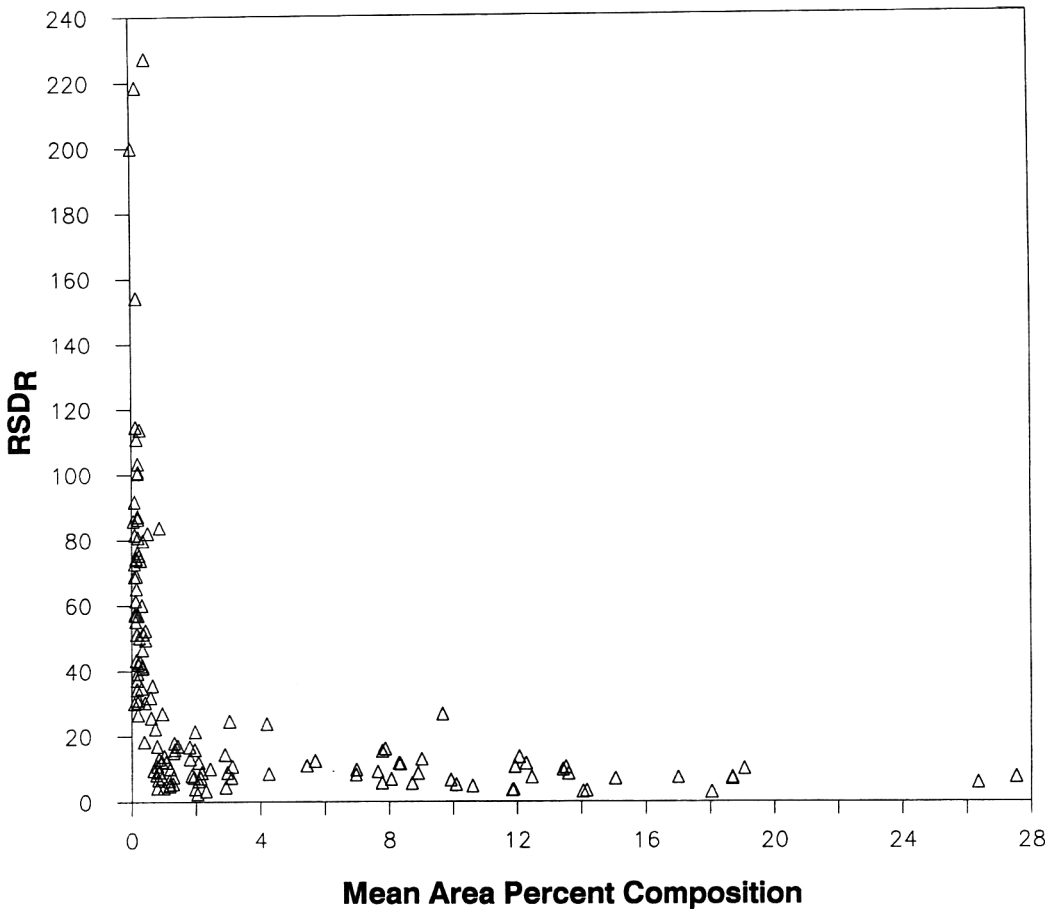

Figure 3. Reproducibility relative standard deviation (RSD_R) for determination of 24 analytes in 5 encapsulated fish oils and 1 ethyl ester concentrate as function of mean area percent composition.

properly, they find it almost impossible to obtain the theoretical factors. This occurred during the collaborative study for unknown reasons. They applied correction factors of 1.03 and 1.05 for EPA and DHA, respectively, to obtain the values reported for the study. This collaborator believes that one or more factors other than the column can affect the results of an analysis, but these factors were not identified.

Collaborator 14 submitted a secondary table of 16 additional analytes in the samples, in addition to the 24 components designated on the data sheet. In his view, the methylation procedure is unnecessarily complicated by the requirement for frequent nitrogen flushing because he believes the acids are unlikely to oxidize during their conversion to esters.

Collaborator 15 commented that the use of the suggested theoretical detector correction factors of 0.99 and 0.97 for EPA and DHA, respectively, gives substantially low values of these materials in fish oils and esters although these factors were used in preparing his report. This laboratory considers that more appropriate correction factors, relative to the internal standard (23:0), would be 0.80 for EPA and 0.68 for DHA. Another comment concerned the small volume of sample solution obtained. This presented a problem because this laboratory uses autosampler vials that require about 1.1 mL sample per injection.

Collaborator 17 raised 3 questions: (*1*) Why is there an apparent large difference between their area percent composition and their calculated weight composition? (*2*) Is 23:0 the most appropriate internal standard, given its relatively poor solubility in many organic solvents? (*3*) What are the relative merits of on-column vs split injection?

Discussion

Conventional GC on packed columns measures the proportions of individual fatty acids after their conversion from triacylglycerol form to volatile methyl ester form (5). However, this approach has several shortcomings. Packed columns are capable of resolving about 12 major or biochemically interesting fatty acids of fish oils (6), but fish oils contain 60 or more constituent fatty acids (7, 8). This limited resolving power of the polar packed GC columns formerly in common use may result in coelution of other components with EPA and/or DHA, exaggerating their respective percentages. This problem of peak coincidence was addressed earlier by Ackman when he described the chain length overlap that is common with packed polar columns (9). As an example, methyl docosenoate (22:1) coelutes with methyl arachidonate (20:4n-6) on the most highly

Table 10. Precision of capillary column analysis of encapsulated marine oil fatty acid esters

	Composition, area %													
	Sample[a]													
	1		2		3		4		5		6		1 and 6	
Fatty acids	Mean	RSD_R, %[b]	Mean	RSD_R, %	Mean	RSD_R, %	Mean	RSD_R, %	Mean	RSD_R, %	Mean	RSD_R, %	Mean	RSD_r, %[c]
14:0	8.3	11.7	0.4	17.1	5.7	13.1	7.0	8.3	5.4	12.0	8.3	11.6	8.3	5.8
16:0	18.7	7.0	0.9	12.2	13.3	7.5	15.0	6.9	7.8	5.7	19.0	10.0	18.7	2.9
16:1	12.1	13.6	0.3	31.0	8.8	7.5	8.1	6.8	9.7	26.7	11.9	10.7	12.0	4.3
18:0	3.1	3.6	2.1	7.0	2.3	3.1	2.9	14.4	0.8	5.6	3.1	5.7	3.1	1.8
18:1	11.9	1.9	8.7	5.7	18.0	2.8	14.1	3.0	10.2	6.7	11.9	3.6	11.9	1.6
18:4n-3	2.9	2.4	1.9	7.6	2.0	4.2	2.1	6.3	4.4	5.5	2.9	4.5	2.9	1.5
20:1	1.8	8.0	14.2	3.2	10.0	4.5	3.2	9.7	1.3	18.1	1.8	16.2	1.8	7.3
20:5n-3	13.8	5.8	26.4	5.6	7.8	5.5	17.0	7.4	27.5	7.5	13.4	9.8	13.8	1.9
22:1	1.4	17.2	10.6	4.7	9.0	8.9	2.4	10.0	1.0	13.4	1.4	6.1	1.4	7.9
22:5n-3	2.0	14.6	4.3	8.5	1.0	9.7	2.1	12.2	2.2	8.8	2.0	16.2	2.0	6.7
22:6n-3	7.9	16.1	18.7	7.5	7.0	9.9	12.3	11.7	12.5	7.5	7.8	15.5	7.9	3.7
	mg/g sample													
20:5n-3	115.3	6.0	223.1	9.2	64.3	7.7	157.5	19.8	241.4	5.4	117.7	7.9	116.5	5.9
22:6n-3	66.0	4.2	157.9	9.0	56.4	8.3	105.3	12.6	105.4	5.2	67.2	8.6	66.6	5.3

[a] Soft gelatin encapsulated samples: 1, steam-deodorized menhaden oil; 2, commercial ethyl ester preparation; 3, cod liver oil; 4, commercial fish oil; 5, commercial fish oil enriched in EPA and DHA; 6, steam-deodorized menhaden oil (blind duplicate of Sample 1).

[b] Reproducibility relative standard deviation.

[c] Repeatability relative standard deviation.

polar columns but with methyl EPA on less polar columns (6, 9). These particular GC problems can be overcome by the use of polar capillary columns, particularly the relatively low polarity polyglycol (Carbowax-20M) columns that have provided for nearly 2 decades maximum resolution of a large number of methyl esters of fatty acids without chain length overlap (10, 11). A typical chromatogram of menhaden oil fatty acid methyl esters on Carbowax-20M is illustrated in Figure **991.39**.

Another problem in the determination of EPA and DHA in marine oils arises from the complex nature of the sample itself. In many analyses of fats and oils, it is conventional to regard the area percentages of fatty acid methyl esters as representative of the mass of the fatty acids in the oil sample; the glycerol content is generally ignored. However, unrefined fish oils contain at least 0.5% free sterols, mostly cholesterol in all instances (12), and, depending on origin, may also contain biogenic hydrocarbons, such as pristane or squalene (13), or fatty alcohols (14). Hexadecanol and docosenols are only 2 examples of the latter, which may be present at as much as 1% of the total lipid content (15). Although some of the more volatile impurities may be removed during steam deodorization, the final step in oil refining (16, 17), this does not include stearyl esters or wax esters (14), also commonly found in marine oils. Unfortunately, during deodorization, the labile EPA and DHA may form either thermal artifacts (18) or nonvolatile oxidative polymers (19). Moreover, companies producing encapsulated fish oils may add tocopherols and sometimes other materials as antioxidants or stabilizers (20). Because polymers, or other nonvolatile materials, are not eluted during GC analysis, the apparent proportions of EPA and DHA are further inflated above the true values, unless properly expressed as mg/g sample. The problems presented by the presence of naturally occurring nontriacylglycerol components or manufacturing additives in encapsulated fish oils require the use of an internal standard. Tricosanoic acid (23:0) has been suggested (21, 22) and tested with both capillary and packed columns. A concerted attack on the problem (23) showed that methyl 23:0 did not coelute with any of the fish oil fatty acid methyl esters when analyzed on a flexible fused silica column coated with a bonded liquid-phase based on the polyglycol, Carbowax-20M (Figure **991.39**). The option of calibrating the EPA and DHA peaks against external standard esters must be mentioned, but it should be discouraged on the grounds of the considerable expense of purified EPA and DHA and the known oxidative instability of these 2 compounds once their containers are opened for use (24–26).

The final problem in the analysis of fish oil fatty acids lies in the need to apply corrections to the electronic response of the universally used flame ionization detector to equate peak areas with mass for the wide range of fatty acid chain lengths, C_{14}–C_{24}, present in fish oils. Although these theoretical detector correction factors (4, 27) are relatively unimportant in the analysis of those vegetable oils that are primarily made up of C_{16} and C_{18} fatty acids (28), they are clearly necessary in the analysis of fish oil fatty acids. Because only the theoretical detector factors should be used, the importance of instrument optimization cannot be overemphasized (4). Capsules of Sample 1 for instrument optimization are available from the Charleston Laboratory, National Marine Fisheries Service, PO Box 12607, Charleston, SC 29422.

A particular benefit of the polyglycols as a recommended liquid phase is that this material is homogeneous in chemical composition, and any bleed with use over time does not change the polarity. Experience indicates that the coating thickness may decrease with use, but the loss of fragments of long molecules does not change the polarity of the balance of the coating. The relative retention times (and resolution) of fatty acids of differing type, especially 21:5n-3 and 23:0, therefore, do not change even if the on-column load has to be slightly reduced with time. Overall, the stability of "bonded" polyglycol columns is such that 2 years of useful life can be expected and is often exceeded. A chemically mixed phase may also lose parts of polymer chains without any effect on polarity, but, in the long run, it appears that a portion of one part, the phenyl groups of DB-225 for example, may be affected, redefining the chemical nature of the liquid phase and altering elution patterns. The only caveats for use of the readily available commercial polyglycol columns are that neither 24:0 nor 24:1 coelutes with 22:6n-3 and that 21:5n-3 be resolved from 23:0. The mono-*trans* artifacts of EPA (18) do not interfere with its determination on polyglycols and, indeed, are an accurate measurement of sample abuse. Similar artifacts of DHA have not been investigated as thoroughly.

Some lipid chemists use antioxidants in solvents, but less trouble from contamination generally follows from the liberal use of an inert gas, usually nitrogen, to exclude atmospheric oxygen. Most polyunsaturated fatty acids have induction periods before severe autoxidation begins (24, 25). Nevertheless, some sense of urgency should accompany all analyses of fish oils, especially refined oils that have been removed from the natural matrix where carotenoids, squalene, etc., may be natural antioxidants. The procedure described may take only 2 h to complete, but the increasing use of autosamplers with delayed overnight analysis reinforces the need to exclude oxygen at every step in the procedure.

Of 2964 potential values to be reported in this study, 2346 area percent values and 180 weight values were received. A few collaborators consistently failed to list values for 20:0, 20:2n-6, 20:3n-6, and 20:3n-3; other collaborators consistently listed these components as being present at 0.1–0.4%. It is probable that the first group of collaborators operated their instruments at less than adequate sensitivity. Dixon and/or Grubbs tests identified a total of 109 outlier values among the 2526 values submitted. Area percent outlier values were more common in the reports of Collaborators 15, 16, 18, and 20; each reported data containing outliers in 5 of the 6 analyses. Six collaborators reported no values that were outliers. Of the 12 outlier values for weights of EPA and DHA, 10 were submitted by Collaborator 12, although none of the corresponding area percentages reported by this collaborator were outliers. In initially reporting 2500–10 000 mg of EPA and DHA/g sample, Collaborator 12 obviously made calculation errors. However, because no "correc-

tion" factor that would bring the second set of values more in line with those submitted by others is evident, this collaborator may not have completely redissolved the internal standard after addition of the samples and solvent.

The results of the analysis of Samples 1–6 are summarized in Table 9 and compared with the results of 2 prior collaborative studies that included fish oil as a sample (29). Reproducibility relative standard deviations (RSD_R) are listed in this table as coefficients of variation (CV) for consistency with the previously published data. With few exceptions, the CVs of the current study were equivalent to, or substantially lower than, those of the 2 earlier studies.

In Figure 3, 144 mean area percent values (6 samples, 24 analytes) are plotted against the respective RSD_R values to illustrate the observed relationship between the 2 variables in this study. Almost half of these mean values (67) were for fatty acids present at less than 1% of the total acids, and the RSD_R values are elevated, with a cluster between about 25 and 60. These elevated values may be anticipated for fatty acids such as 22:0, 22:4n-6, 22:5n-6, 24:0, and 24:1, which rarely exceed 0.5% in marine oils. The RSD_R values decline to about 10 for means of fatty acids present at 2% or greater. The highest mean observed (27.5%) had an RSD_R of about 5.

The International Standards Organization has introduced the concepts of repeatability confidence value (r) and reproducibility confidence value (R) (3). As stated in the AOAC guidelines for collaborative study procedures, "...assuming normal distribution, when duplicate measurements are performed, the absolute difference between the results of each of these duplicate measurements is expected to be below r or R in 95% of the cases" (30). Thus, these values represent the 95% confidence limits of the differences between 2 successive analyte concentration estimates. In the analysis of blind duplicate Samples 1 and 6 for area percentages of EPA (r = 0.7) and DHA (r = 0.8), only 1 collaborator reported replicated values of EPA that differed by more than 0.7; 2 collaborators obtained results with differences greater than 0.8 for DHA. These aberrant results are clearly seen in Figure 1.

Table 10 summarizes the precision of the capillary column analysis of the more important fatty acids of fish oils, particularly of the nutritionally important n-3 fatty acids. As noted above, the reproducibility for the major fatty acids equals, or is superior to, that obtained in a prior collaborative study of fish oils using packed GC columns.

Recommendation

We recommend that the capillary column GC method for determination of fatty acids of fish oils and ethyl esters derived from that source, both as area percentages and in absolute weights through the use of 23:0 as internal standard, be adopted first action.

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OILS AND FATS

Rapid and Simple Coulometric Measurements of Peroxide Value in Edible Oils and Fats

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A rapid coulometric method was developed for the measurement of peroxide value in edible oils and fats. The sample size and reagents volumes in this method are considerably less than those in the American Oil Chemists' Society method. Iodine produced by the reaction of the iodide ion and peroxide in the sample is electrochemically reduced at the carbon-felt electrode more rapidly than it is with iodometric titration. The present method is successfully applied to the measurements of edible oils and fats, and the coulometric results obtained are consistent with those obtained by iodometry.

The oxidation of lipids in food affects the deterioration of food quality by producing rancidity and inviting food poisoning. Therefore, the oxidation of lipids in food must be investigated when addressing food sanitation.

Peroxide value (POV) was used as an effective indicator of the degree of the oxidation of lipid. The official method of the Japan Oil Chemists' Society (1) and the American Oil Chemists' Society (AOCS) (2) measure POV iodometrically. However, the determination of low levels of POV or POV in small sample sizes is uncertain because the iodometric titration endpoint, the disappearance of the pale-violet color produced by the iodine and starch reaction, is difficult to discern.

A coulometric method was developed to complete accurate measurements of low level POV (3). However, this method is time-consuming.

We developed a rapid, controlled potential coulometric procedure that was applied to the determination of various substances such as L-ascorbate (4), residual chlorine (5), and hydrogen peroxide (6). We also measured the POV of linoleic acid by the rapid coulometric procedure (7). However, this method was not applicable to POV of fats such as lard or beef tallow because the solid-state sample at room temperature was not sufficiently diffused into the working electrode. Therefore, the coulometric analysis for the measurement of POV in oils and fats was improved by modifying the sample preparation of the AOCS method.

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Experimental

Reagents

(a) *Edible oils and fats.*—Sesame, corn, cottonseed, rapeseed, peanut, olive, and palm oil, lard and beef tallow (Yuro Chemical Co., Tokyo, Japan). Oxidized by incubation in the dark at 60°C.

(b) *Acetic acid–chloroform solution (3 + 2).*—Mix glacial acetic acid and chloroform.

(c) *Saturated potassium iodide solution.*—Dissolve potassium iodide in freshly boiled water.

(d) *Electrolyte.*—0.2M Sodium dihydrogenphosphate dihydrate including 1M potassium iodide (pH 3.0).

Apparatus

(a) *Potentiostat.*—Model HA-305 (Hokuto Denko Ltd, Tokyo, Japan).

(b) *Integrator.*—Model C-R2A (Shimadzu, Kyoto, Japan).

(c) *Carbon-felt cell.*—Mitsui Engineering & Ship-building Co., Ltd, Chiba, Japan.

(d) *Automated repeating microsyringe.*—Model MS-5SL (Ito Co., Ltd, Shizuoka, Japan).

(e) *Personal centrifugal separator.*—Model Chibi-tan (Nihon Millipore, Ltd, Tokyo, Japan).

Sample Preparation

Accurately weigh ca 20 mg sample into 0.5 mL disposable polypropylene centrifugal tube. Add 150 μL glacial acetic

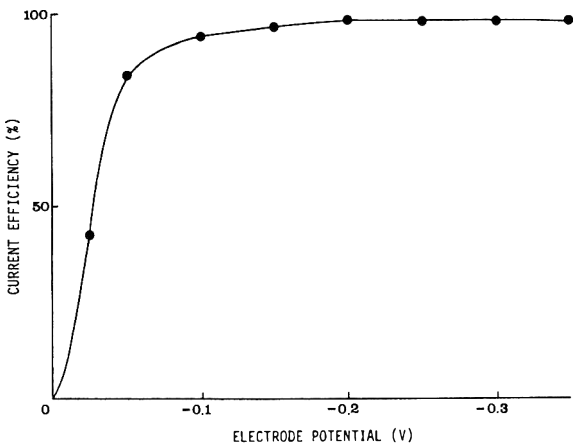

Figure 1. Effect of electrode potential on current efficiency of 1 × 10^{-3}M I_2.

acid–chloroform (3 + 2, v/v) to dissolve sample, and add 2 μL saturated potassium iodide. After occasional mixing on vortex mixer for 1 min, add 150 μL distilled water and shake vigorously. Centrifuge mixture 10 s at 6400 rpm. Discard lower chloroform layer using disposable pipet. Use aqueous layer for test solution.

Measurement of POV

Construction of the coulometric cell presented here is the same as that used in a previous study (8), except for the electrode size and the electrolyte, which is changed to 0.2M sodium dihydrogenphosphate dihydrate containing 1M potassium iodide.

Table 1. Variation of current efficiencies of 1 × 10^{-3}M I_2 obtained by successive injections[a]

No. of injection	Charge, mC	C.E., %	CV, %	E.T., s
1–5	0.993	102.8	0.46	9.5
6–10	0.992	102.7	0.47	10.8
11–15	0.989	102.4	0.13	9.7
16–20	0.987	102.2	0.49	11.8
21–25	0.987	102.2	0.49	10.7
26–30	0.986	102.1	0.67	11.8
31–35	0.977	101.1	0.33	10.8
36–40	0.980	101.4	0.67	13.4
41–45	0.976	101.0	0.86	15.0
46–50	0.972	100.6	0.86	15.4
51–55	0.961	99.5	0.87	15.7
56–60	0.948	98.1	2.25	16.3
61–65	0.941	97.4	2.42	17.9
66–70	0.892	92.3	3.02	17.5

[a] Average of 5 trials: Injection volume, 5 μL; C.E. = current efficiency; CV = coefficient of variation; and E.T. = electrolysis time.

Inject triplicate 5 μL volumes of test solution and blank solution without lipid onto the surface of the carbon-felt cell. Mean values of charges integrated during electrolysis were converted into POV.

Results and Discussion

Electrode Potential

In controlled potential coulometry, the electroactive species must be completely electrolyzed at the controlled potential.

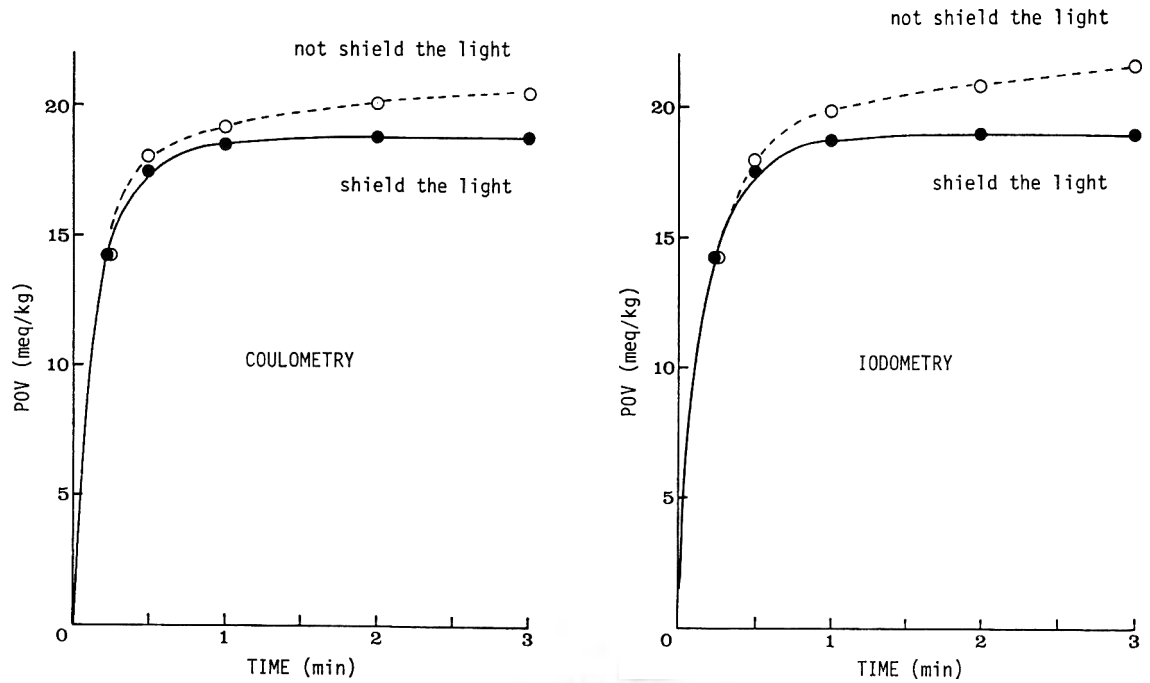

Figure 2. Effect of light on the peroxide, potassium iodide reaction.

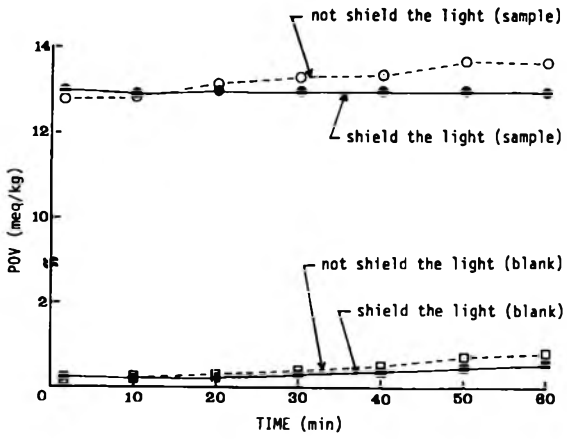

Figure 3. Effect of light on iodide stability.

Table 2. Comparison of POV of edible oils and fats obtained by coulometry and iodometry[a]

Sample	Coulometry		Iodometry[b]	
	POV, meq/kg	CV, %	POV, meq/kg	CV, %
Sesame oil	4.0	2.1	4.1	2.2
Corn oil	8.4	1.8	8.7	0.96
Cottonseed oil	13.8	3.3	14.5	2.7
Rapeseed oil	33.1	3.0	33.2	0.27
Peanut oil	29.2	1.9	30.5	0.18
Peanut oil	35.4	2.9	37.8	0.85
Olive oil	16.0	0.36	17.0	0.67
Olive oil	49.6	1.6	50.3	0.14
Palm oil	8.9	0.94	8.9	0.94
Beef tallow	2.5	7.8	2.5	1.8
Lard	35.0	0.82	34.6	0.72

[a] Average of 5 trials; CV = coefficient of variation.
[b] AOCS method (shield the light).

Current efficiency against applied potential was obtained by injecting the iodide solution (5 μL, 10^{-3}M) at different electrode potentials. Current efficiency increased with an increase in working electrode potential, and efficiency reached a maximum value at more than –0.20 V (Figure 1). However, the electro-reductions of other electroactive species contained in the sample were observed when the electrode potential exceeded –0.3 V. Consequently, a –0.25 V electrode potential of the working electrode was selected.

Sample Load

The effect of successive injections of 1×10^{-3}M iodine on current efficiency was studied. Data in Table 1 show that more than 50 injections were permitted. The increase of volume of electrolyte due to successive injections possibly delayed the diffusion rate of iodine into the electrode, and this delay may have resulted in the low current efficiency. Rapid and accurate sampling was performed with an automated repeating microsyringe.

Linearity and Detection Limit

The relationship between charge and iodine concentration was linear over the $2 \times 10^{-2} - 1 \times 10^{-5}$M range, which is equivalent to a POV of 482–0.28 meq/kg, with a correlation coefficient of 0.9999. The detection limit of POV was defined as 3 times the blank run because the higher blank run coefficients of variation influence the sample coefficients of variation. The detection limit was about 0.8 meq/kg.

Effect of Light on POV

The effects of light on POV during and after the peroxide and potassium iodide reaction are shown in Figures 2 and 3, respectively. These results indicate that the liberation of iodine was increased by light; however, this reaction was completed within 1 min by shielding the light. Therefore, the method should be performed with light shielding. Because the POV of the blank solution is stable for 20 min after production of iodine, injections should be performed within this time period.

Application to Oils and Fats

The present method was applied to POV measurements of several oils and fats, and the results were compared to those from the AOCS method (2) (Table 2). Except for a few samples, coulometric results were slightly lower than those ob-

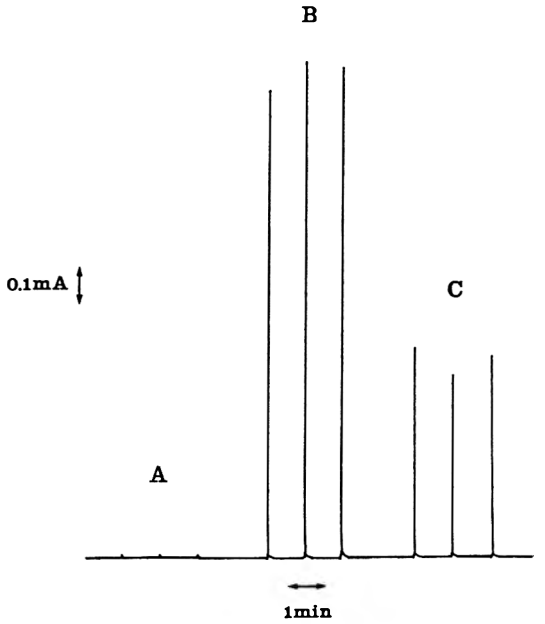

Figure 4. Typical current vs time curves obtained from blank solution and oxidized lards: A, blank solution; B, lard (POV 17.9 meq/kg); and C, lard (POV 8.7 meq/kg).

tained by the AOCS method. This indicates that iodine is extracted incompletely from the organic phase to the aqueous phase. Typical current vs time curves obtained from oxidized lard samples and blank solutions are shown in Figure 4. The electrochemical reduction of each sample is complete within about 20 s.

The rapid coulometric method was applied for measurement of POV in oil (7). In this method, the sample solution containing oil was directly injected into the working electrode; however, the solid sample at room temperature was diffused incompletely into the working electrode. In the present study, modifications of the sample preparation, the electrolytic potential, or the electrolytic solution made the detection of POV in solid fat samples possible. Therefore, the present method is more applicable for rapid and precise measurement of POV in various edible oils and fats.

Acknowledgments

The authors thank Huminori Satoh for his assistance in the development of this method, and Masaharu Takabatake of Mitsui Engineering & Ship-building Co., Ltd, Chiba, Japan, for supplying the coulometric cell with carbon-felt electrode.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Multiresidue Method for Quantitation of Organophosphorus Pesticides in Vegetable and Animal Foods

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A critical review of multiresidue analytical methods was performed to develop an easy procedure that can be applied with slight modifications to vegetable and animal food samples. The multiresidue method described is applicable to the quantitative determination of 28 organophosphorus pesticides in fatty as well as nonfatty foods. The foods are divided into 4 groups according to extraction process (acetone and acetone–water for samples with moisture content <45%) and cleanup (on active carbon–Celite, on disposable minicolumns of Kieselguhr-type material, and on disposable minicolumns of bonded-phase silica, according to fat and pigment content). A further fractionation on silica-gel microcolumns can be included in the procedure. A wide-bore column (SPB-5) with flame photometric detection operated in phosphorus mode (FPD-P) was chosen for gas chromatographic determination. Recoveries from apples, olive oil, whole milk, pasta, and eggs are reported. Two packed columns (4% OV-101 and SP2250–SP2401) with FPD-P were tested as confirmation columns. The method is suitable for a total diet study.

Organophosphorus pesticides are the most widely used insecticides in Italy: 19 053 tons of organophosphorus insecticides out of a total 33 025 tons of organic insecticides were used for agriculture during 1987 (1).

The Ministry of Health commissioned the Hygiene Institute of the University of Rome to perform a total diet study to determine the organophosphorus pesticide intake.

The number of samples analyzed is an important determinant of the reliability of a total diet study. Thus, the analytical procedure must be fast, easy, applicable with slight modifications to a number of different sample classes, and sufficiently sensitive and selective.

Well-known multiresidue methods were studied, checked, and integrated. The relevant aspects selected from these published methods to be applied to the determination of organophosphorus pesticides in foods are shown in Table 1.

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The multiresidue analytical method selected for the determination of 28 organophosphorus insecticides in individual vegetable and animal foods is described. It is based in particular on the works of Ambrus et al. (2), Blaha and Jackson (3), Luke and Doose (4), and Di Muccio et al. (5). The insecticides were selected on the basis of the Italian regulations (6, 7) and volume and distribution of use (1).

Experimental

Apparatus and Reagents

(a) *Gas chromatographs.*—(*1*) Varian Model 3700 (Walnut Creek, CA) equipped with: flame photometric detector operated in phosphorus mode (FPD-P), 526 nm filter. Operating conditions: injector 220°C, detector 250°C; establish stable flame at electrometer setting that will produce 40% full-scale deflection for 1 ng parathion-methyl; baseline noise should be <2%. Columns: (*a*) SPB-5 (Supelco, Bellefonte, PA), 30 m × 0.53 mm id; hold at 140°C for 2 min, increase to 240°C at 5°C/min and hold 2 min; N_2 carrier gas, 15 mL/min; N_2 makeup, 15 mL/min. (*b*) Glass, 2 m × 3 mm id, packed with 4% OV-101 on 80–100 mesh Supelcoport (Supelco); 170°C, increase to 250°C at 5°C/min and hold 2 min; N_2 carrier gas, 45 mL/min. (c) Glass, 2 m × 3 mm id, packed with 1.5% SP2250 + 1.95% SP2401 on 100–120 mesh Supelcoport (Supelco), hold at 175°C for 2 min and increase to 240°C at 5°C/min and hold 10 min; N_2 carrier gas, 45 mL/min. (*2*) Hewlett-Packard Model HP 5890 (Palo Alto, CA) equipped with: MSD 5970 B. Operating conditions: SIM mode, EI 70 eV, splitless injector 220°C. Column: ULTRA-2 (HP), 25 m × 0.2 mm id, 0.11 μm film thickness; hold at 80°C for 2 min and increase to 140°C at 20°C/min, and then to 250°C at 3°C/min; He carrier gas, 65 kPa.

(b) *Chromatographic cleanup columns.*—(*1*) Ready-to-use Extrelut-3 (Cat. No. 15327, Merck, Darmstadt, Germany); fix needle (Luer-Lock 0.65/32) at column end as flow regulator. (*2*) Glass, 30 cm × 20 mm id with glass septum (carbon–Celite cleanup). (*3*) Glass microcolumn, 30 cm × 4.2 mm id, with a 25 cm × 20 mm id reservoir, joined at an air inlet, for chro-

Table 1. Extraction and cleanup procedures from published methods to determine organophosphorus residues in food

Type of food	Extraction	Cleanup		No. of pesticides analyzed	Reference
		Solvent partition	Further processing		
Dry crop, low fat content	Methylene chloride	Acetone–water	Alumina, silica gel	36	(4)
Dry vegetables (<10% water)	Acetone–water (65%)	Methylene chloride–acetone, sodium chloride	Carbon–Celite	7	(6)
Fatty foods	Acetone–benzene–sulfuric acid	Any	Fat precipitation at low temperature	19	(15)
Fatty and nonfatty foods	Acetone, acetone–water	Methylene chloride–hexane, methylene chloride–acetone–sodium chloride	GPC	17	(5)
Fruits and vegetables	Acetone	Methylene chloride	Alumina, silica gel, mixed adsorbent	36	(4)
		Methylene chloride–petroleum ether	Florisil	15	(12)
		Methylene chloride, hexane	Any	46	(15)
		Methylene chloride, hexane	Florisil	16	(15)
		Methylene chloride	Silica gel, carbon	43	(17)
Milk	Acetone	Methylene chloride	Any	20	(11)
Milk and cream	Acetone–hexane	Any	Florisil	1	(15)
Nonfatty foods	Acetonitrile, acetonitrile–water	Methylene chloride	Mixed adsorbent	41	(14)
Olive oil	Any	Any	Minicolumn Kieselguhr-type material	9	(7)
Unpolished rice, wheat, buckwheat, dried beans	Acetone, benzene, hexane, acetone–benzene	Acetonitrile	Carbon	11	(10)
Water	Methylene chloride	Any	Silica gel	19	(8–9)
Wheat	Ethyl ether–hexane	Any	Any	15	—[a]

[a] Levi, I., & Nowicki, T.W. (1974) *J. Assoc. Off. Anal. Chem.* **57**, 924–929.

matography on silica gel (8, 9). (*4*) Ready-to-use Supelclean LC-18 (Supelco, Cat. No. 5-7012).

(c) *Chopper, grinder.*—Domestic machine.

(d) *High-speed blender.*—Omni-mixer (Omni International, Waterbury, CT).

(e) *Rotary vacuum evaporator.*—Operate at 90 rpm (Buchi, Switzerland).

(f) *Reference standard materials.*—Acephate, azinphos-ethyl, azinphos-methyl, bromophos, chlorfenvinphos E and Z, chlorpyriphos and chlorpyriphos-methyl, demeton-*O*, demeton-*S*-methyl, demeton-*S*-methylsulfone, diazinon, dimethoate, ethion, fenitrothion, heptenophos, malaoxon and malathion, methamidophos, methidathion, monocrotophos, omethoate, paraoxon, paraoxon-methyl, parathion, parathion-methyl, phosalone, pirimiphos-methyl, tetrachlorvinphos, and vamidothion (Società Italiana Chimici S.p.A., Italy). Prepare individual standard solutions in *n*-hexane at 100–300 µg/mL concentration, adding benzene (ca 1%), if solubilization is difficult; then prepare suitable dilution with *n*-hexane. Prepare standard cumulative solutions (for recovery test) by mixing suitable volumes of individual standard solutions and diluting with *n*-hexane.

(g) *Solvents.*—Acetone, acetonitrile, benzene, dichloromethane, methanol, *n*-hexane, all pesticide grade (Carlo Erba, Milan, Italy). Handle solvents, particularly benzene, under a fume hood, avoiding inhalation and contact with skin. Keep away from sources of ignition.

(h) *Anhydrous sodium sulfate.*—Treated at 550°C for 16 h (Carlo Erba).

(i) *Silanized glass wool.*—(Applied Science Laboratories, State College, PA).

(j) *Celite-545.*—0.020–0.045 mm (Serva, Seinbionchemica, Heidelberg, Germany, and New York, NY).

(k) *Active carbon.*—Darco G-60 (Fisher, Fairlawn, NJ).

(l) *Sodium chloride.*—(Carlo Erba).

(m) *Silica gel.*—Davison 923, treated at 130°C for 2 h and, after cooling, deactivated by 6.5% distilled water (BDH Ltd, Poole, England).

(n) *Cotton wool.*—Washed with acetone and *n*-hexane in Soxhlet and air dried.

Grouping of Food Samples

Foods are divided into 4 main groups according to moisture and fat content. This subdivision with the adopted extraction process and cleanup for each group is shown in Table 2.

Extraction

(a) *Groups I, II, and IIa.*—Weigh 50 g chopped sample into high-speed blender jar, add 100 mL acetone, blend 2 min at high speed. Filter with suction through Buchner funnel with glass septum, wash residue with ca 50 mL acetone. Collect washing and add to filtrate. Bring extract to an exact volume (150–200 mL) with acetone–water (2 + 1).

(b) *Groups III (except oil) and IV.*—Weigh 50 g chopped sample into high-speed blender jar, add 50 mL distilled water, and proceed as for Groups I and II.

Table 2. Grouping of food samples for extraction and cleanup

Group	Content, %		Type of food	Extraction	Solvent partition	Cleanup
	Water	Fat				
I	> 45	< 2	Vegetables, fresh fruits	Acetone	Methylene chloride–sodium chloride	Celite–active carbon
II	> 45	> 2	Whole milk, green cheese	Acetone	Methylene chloride–sodium chloride	Extrelut-3
IIa	> 45	> 2	Eggs, meat	Acetone	Methylene chloride–sodium chloride	Extrelut-3–Supelclean LC-18
III	< 45	> 2	Cheese, oil, dried legumes	Acetone–water (except oil)	Methylene chloride–sodium chloride (except oil)	Extrelut-3
IV	< 45	< 2	Wheat meal, pasta, rice, bread	Acetone–water	Methylene chloride–sodium chloride	Celite–active carbon

Table 3. GC retention times (min) of organophosphorus insecticides relative to parathion-methyl

Compound	Column		
	SPB-5[a]	OV-101[b]	SP2250–SP2401[c]
Acephate	0.26	—[d]	—[d]
Azinphos-ethyl	2.29	3.14	2.91
Azinphos-methyl	2.16	2.83	2.66
Bromophos	1.24	1.41	1.07
Chlorfenvinphos E	1.31	1.47	1.24
Chlorfenvinphos Z	1.35	1.55	1.32
Chlorpyriphos	1.18	1.30	0.97
Chlorpyriphos-methyl	0.99	1.00	0.82
Demeton-*O*	0.48	0.45	0.60
Demeton-*S*-methyl	0.49	0.44	0.56
Demeton-*S*-methylsulfone	1.09	—[d]	0.13
Diazinon	0.84	0.81	0.55
Dimethoate	0.69	0.64	0.89
Ethion	1.73	2.18	1.69
Fenitrothion	1.11	1.16	1.09
Heptenophos	0.42	0.39	0.46
Malaoxon	1.03	1.04	1.19
Malathion	1.16	1.22	1.09
Methamidophos	0.09	—[d]	—[d]
Methidathion	1.40	1.69	1.46
Monocrotophos	0.64	0.66	1.12
Omethoate	0.48	0.52	0.90
Paraoxon	1.06	0.82	1.21
Paraoxon-methyl	0.85	0.84	1.08
Parathion	1.19	1.30	1.16
Parathion-methyl	1[e]	1[f]	1[g]
Phosalone	2.20	2.90	2.45
Pirimiphos-methyl	1.11	1.58	0.92
Tetrachlorfenvinphos	1.45	1.19	1.46
Vamidothion	1.45	—[d]	1.87

[a] Fused silica, 30 m × 0.53 mm id; hold at 140°C for 2 min, increase to 240°C at 5°C/min and hold 2 min; N_2 carrier gas, 15 mL/min.
[b] Glass, 2 m × 3 mm id, packed with 4% OV-101 on 80–100 mesh Supelcoport; 170°C, increase to 250°C at 5°C/min and hold 2 min; N_2 carrier gas, 45 mL/min.
[c] Glass, 2 m × 3 mm id, packed with 1.5% SP2250 + 1.95% SP2401 on 100–120 mesh Supelcoport; hold at 175°C for 2 min, increase to 240°C at 5°C/min and hold 10 min; N_2 carrier gas, 45 mL/min.
[d] Compound is not revealed at these operating conditions.
[e] 9.20 min.
[f] 5.15 min.
[g] 8.25 min.

Partition

For all groups (except oil), place half the volume of food extract, equivalent to 25 g of sample (reserve second half) in a separatory funnel and add 100 mL dichloromethane, 100 mL acetone, and ca 7 g sodium chloride (to saturate aqueous solution). Shake vigorously 1 min until most sodium chloride is dissolved, allow layers to separate, and transfer aqueous layer (lower layer) to a second separatory funnel. Dry organic layer (upper layer) through sodium sulfate, about 50 g placed in a glass column 15 cm × 3.5 cm id, with a plug of cotton wool at the bottom. Add two 100 mL portions of dichloromethane to second separatory funnel and shake vigorously 1 min each time, and then dry each organic layer (lower layer) as above. Rinse sodium sulfate with ca 50 mL dichloromethane. Collect organic layers and washing and concentrate just to dryness in rotary evaporator (40–45°C water bath, reduced pressure). Add suitable solvent for chromatographic cleanup.

Column Chromatographic Cleanup

(a) *Groups I and IV.*—Fill glass column (2 cm id, glass septum) with 2 g Celite followed by 4 g carbon–Celite (1 + 4) and top with glass-wool plug. Wash column with 20 mL benzene. Transfer sample quantitatively to column with small portion of benzene (ca 2 mL) and elute pesticides with 60 mL acetonitrile–benzene (1 + 1). Concentrate just to dryness in rotary evaporator (45–50°C water bath, reduced pressure). Add a suitable volume (1 mL) of benzene and analyze by GC.

(b) *Groups II and III (except oil).*—Transfer sample quantitatively to disposable Extrelut-3 minicolumn with ca 3 mL *n*-hexane. Allow solution to drain into filling material. Wait 10 min to obtain even distribution, then elute 3 times with 5 mL acetonitrile equilibrated with *n*-hexane. Add 4 mL methanol to eluate and concentrate just to dryness in rotary evaporator (50–55°C water bath, reduced pressure). Add suitable volume (1 mL) of benzene and analyze by GC.

(c) *Group IIa.*—Perform cleanup as for Groups I and IV, then quantitatively transfer sample to Supelclean LC-18 with ca 2 mL acetonitrile and elute with 7 mL acetonitrile. Concentrate to dryness in rotary evaporator (50–55°C water bath, reduced pressure). Add suitable volume (1 mL) of benzene and analyze by GC.

(d) *Olive oil.*—Dissolve and dilute olive oil or other vegetable oil (maximum volume 2.5 mL) to 3 mL with *n*-hexane. Quantitatively transfer lipid solution to disposable Extrelut-3 minicolumn, and perform cleanup as for Groups II and III.

Determination

Check that GC systems are working properly by injecting mixed standard solutions. Retention times relative to parathion-methyl, chosen as reference substance, are reported in Table 3 for SPB-5, OV-101, and SP2250–SP2401 columns. Figure 1 shows the chromatographic separation of mixed standard solutions on the SPB-5 column. Inject 1 μL of cleaned extract (equivalent to 25 mg sample) into the SPB-5 column. Check that no peak is present in the retention window of parathion-methyl, and add parathion-methyl to the sample as time reference compound. If there are some peaks, heptenophos can be used as time reference compound. Reinject sample and identify peaks by their relative retention times. Inject sample into SP2250–SP2401 column for confirmation and resolution of overlapping peaks, because this column displays a distinct elution sequence.

Quantify residues by height or by area measurement with a calibration graph obtained daily from solution of known con-

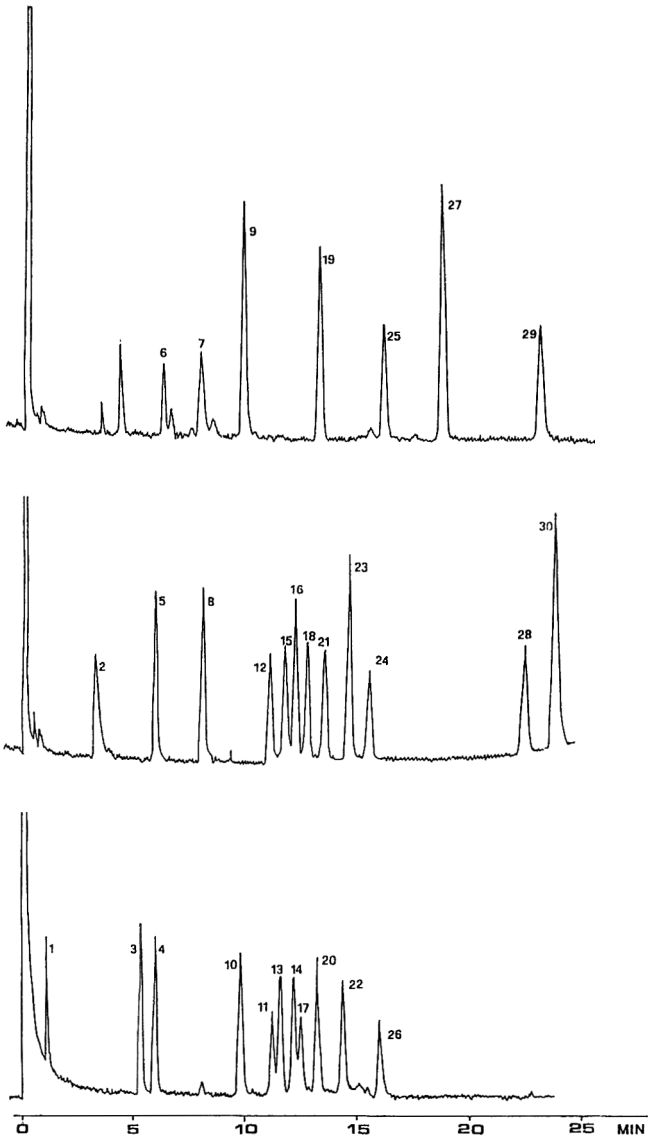

Figure 1. Chromatograms of organophosphorus standard solutions under the following GC conditions: SPB-5 column, with FPD flame photometric detection operated in P-mode; held at 140°C for 2 min, increased to 240°C at 5°C/min and held 2 min. (1) Methamidophos, (2) acephate, (3) heptenophos, (4) demeton-*O*, (5) demeton-*S*-methyl, (6) omethoate, (7) monocrotophos, (8) dimethoate, (9) diazinon, (10) paraoxon-methyl, (11) chlorpyriphos-methyl, (12) parathion-methyl, (13) malaoxon, (14) demeton-*S*-methylsulfone, (15) paraoxon, (16) fenitrothion, (17) pirimiphos-methyl, (18) malathion, (19) chlorpyriphos, (20) parathion, (21) bromophos, (22) chlorfenvinphos E, (23) chlorfenvinphos Z, (24) methidathion, (25) tetrachlorvinphos, (26) vamidothion, (27) ethion, (28) azinphos-methyl, (29) phosalone, (30) azinphos-ethyl.

centrations of the authentic compounds. Table 4 shows the detection limits of organophosphorus insecticides (signal-to-noise ratio = 3) on SPB-5 with FPD detection.

Table 4. Detection limits for organophosphorus insecticides[a]

Compound	Detection limit, ng
Azinphos-ethyl	0.21
Azinphos-methyl	0.45
Bromophos	0.12
Chlorfenvinphos E	0.23
Chlorfenvinphos Z	0.18
Chlorpyriphos	0.13
Chlorpyriphos-methyl	0.10
Demeton-*O*	0.07
Demeton-*S*-methyl	0.09
Demeton-*S*-methylsulfone	1.18
Diazinon	0.13
Dimethoate	0.21
Ethion	0.14
Fenitrothion	0.18
Heptenophos	0.09
Malaoxon	0.29
Malathion	0.18
Methidathion	0.17
Monocrotophos	0.55
Omethoate	0.10
Paraoxon	0.15
Paraoxon-methyl	0.21
Parathion	0.26
Parathion-methyl	0.12
Phosalone	0.31
Pirimiphos-methyl	0.11
Tetrachlorvinphos	0.20
Vamidothion	1.31

[a] SPB-5 column with FPD-P (signal-to-noise ratio = 3; 1 ng parathion-methyl = 40% FSD).

Recovery Test

The foods selected for the recovery tests are apples, whole milk, eggs, olive oil, and pasta for Groups I, II, IIa, III, and IV, respectively. They were chosen because of their high consumption and to demonstrate the applicability of the procedure to different product matrixes. Chopped solid samples and olive oil and milk were spiked at the levels indicated in Table 5, after checking for the absence of organophosphorus. They were subjected to the whole procedure and determined by means of SPB-5 with GC/FPD. Each result (Table 5) is the average of 3 tests.

Results and Discussion

Acetone and acetone–water blending is the most widely used (2, 3, 4, 10–13), simplest, and most efficient method to quantitatively remove the organic chemical residues from foodstuffs. We used acetone–water (2 + 1) for samples with a water content <45% to facilitate the partition step.

For the first purification step, we chose a simple partition with dichloromethane (2, 4, 11, 14). Solvent partitioning of or-

Table 5. Recovery of pesticides from fortified foods[a]

Compound	Apples			Whole milk			Olive oil			Pasta			Eggs		
	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., %
Azinphos-ethyl	0.30	0.27	89	0.29	0.21	71	0.65	0.63	97	0.027	0.024	89	0.058	0.053	91
Azinphos-methyl	0.19	0.18	96	0.18	0.19	103	0.63	0.64	101	0.029	0.027	93	—	—	—
Bromophos	0.21	0.19	89	0.20	0.18	89	0.51	0.44	87	0.028	0.021	75	—	—	—
Chlorfenvinphos E	0.35	0.25	72	0.34	0.33	97	0.66	0.67	101	0.023	0.019	83	—	—	—
Chlorfenvinphos Z	0.38	0.34	89	0.37	0.35	94	0.84	0.76	91	—	—	—	0.021	0.020	97
Chlorpyriphos	—	—	—	—	—	—	—	—	—	0.028	0.022	79	0.033	0.027	80
Chlorpyriphos-methyl	0.20	0.14	69	0.19	0.16	87	0.48	0.46	97	0.030	0.022	73	—	—	—
Demeton-*O*	0.28	0.14	49	0.27	0.16	59	0.39	0.16	42	0.028	0.016	57	—	—	—
Demeton-*S*-methyl	0.18	0.10	54	0.17	0.13	75	0.52	0.13	26	—	—	—	—	—	—
Demeton-*S*-methylsulfone	0.42	0.41	98	0.10	0.08	83	1.21	1.16	96	—	—	—	—	—	—
Diazinon	—	—	—	—	—	—	—	—	—	0.030	0.024	80	0.013	0.012	93
Dimethoate	0.20	0.18	90	0.19	0.18	95	0.43	0.41	96	0.030	0.023	77	0.032	0.025	80
Ethion	—	—	—	—	—	—	—	—	—	0.022	0.020	91	—	—	—
Fenitrothion	0.21	0.19	89	0.20	0.18	89	0.47	0.42	90	—	—	—	—	—	—
Heptenophos	0.31	0.22	71	0.30	0.21	69	0.59	0.56	95	—	—	—	0.020	0.015	77
Malaoxon	0.30	0.25	83	0.29	0.22	76	0.66	0.67	116	—	—	—	0.043	0.035	81
Malathion	0.28	0.25	89	0.27	0.25	92	0.67	0.60	89	0.034	0.028	82	—	—	—
Methidathion	—	—	—	—	—	—	—	—	—	0.036	0.031	86	0.038	0.032	84
Monocrotophos	—	—	—	—	—	—	—	—	—	0.040	0.016	40	—	—	—
Omethoate	—	—	—	—	—	—	—	—	—	0.046	0.031	67	—	—	—
Paraoxon	0.18	0.17	97	0.17	0.15	89	0.48	0.43	90	—	—	—	—	—	—
Paraoxon-methyl	0.30	0.23	75	0.29	0.20	68	0.61	0.58	96	0.040	0.032	80	—	—	—
Parathion	0.32	0.23	71	0.31	0.26	85	0.53	0.52	98	—	—	—	0.017	0.014	80
Parathion-methyl	0.23	0.20	87	0.22	0.19	87	0.58	0.49	85	—	—	—	—	—	—
Phosalone	—	—	—	—	—	—	—	—	—	0.031	0.034	110	0.066	0.056	85
Pirimiphos-methyl	0.20	0.15	73	0.05	0.04	—	0.58	0.56	97	0.027	0.029	107	—	—	—
Tetrachlorvinphos	—	—	—	—	—	—	—	—	—	0.038	0.034	90	—	—	—
Vamidothion	0.29	0.30	103	0.28	0.21	74	1.26	1.01	80	—	—	—	—	—	—
$\bar{X}$	0.27	0.22	82	0.23	0.19	83	0.63	0.56	89	0.031	0.025	81	0.034	0.029	85

[a] Each result is based on an average of 3 tests analyzed by GC/FPD-P on SPB-5 column.

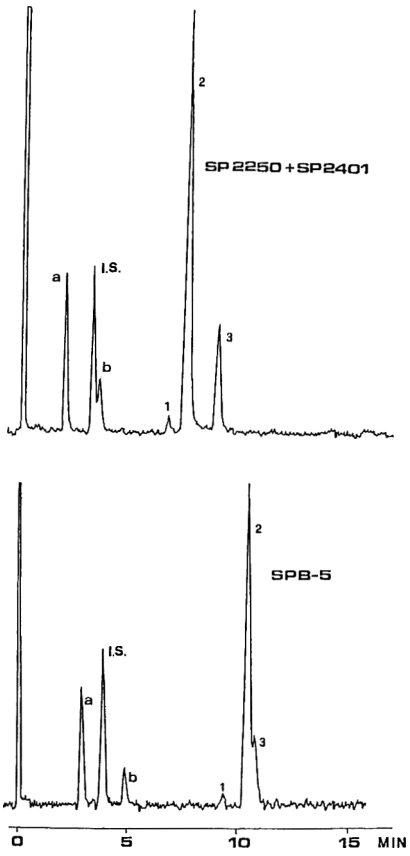

Figure 2. Chromatograms of a bread extract (4 mL final volume) under the following GC conditions: injection of 1 μL on SPB-5 with FPD operated in P-mode; held at 140°C for 2 min, increased to 240°C at 5°C/min and held 2 min. Injection of 5 μL on 1.5% SP2250+1.95% SP2401 with FPD operated in P-mode; held at 175°C for 2 min and increased to 240°C at 5°C/min and held 10 min. (1) Chlorpyriphos-methyl, (2) pirimiphos-methyl, (3) malathion, (a and b) unidentified peaks, and (I.S.) heptenophos as internal standard.

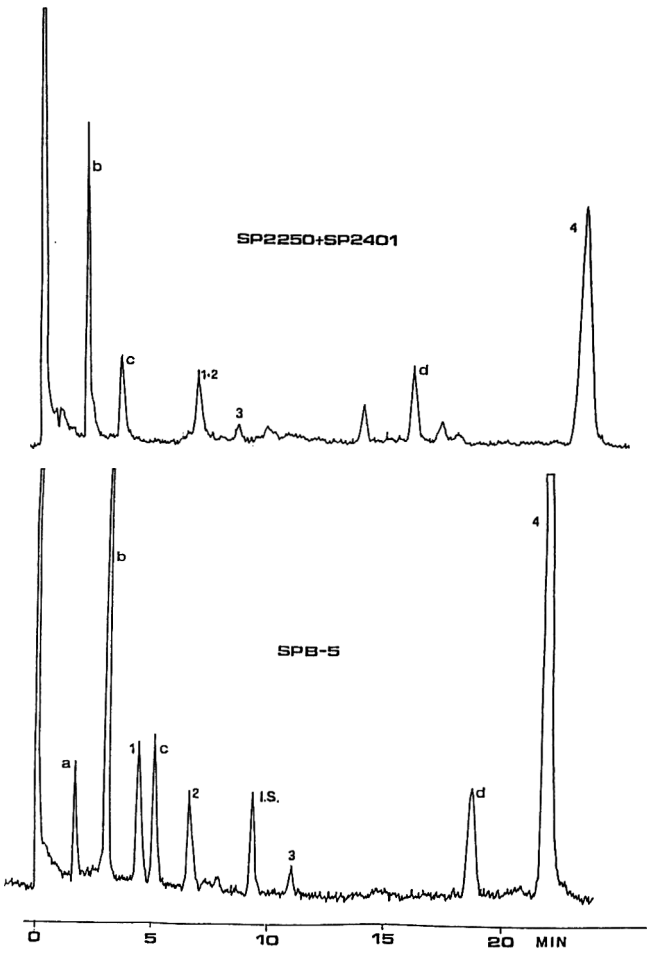

Figure 3. Chromatograms of a lettuce extract (1 mL final volume) under the following GC conditions: injection of 2 μL on SPB-5 with FPD operated in P-mode; held at 140°C for 2 min, increased to 240°C at 5°C/min and held 2 min. Injection of 5 μL on 1.5% SP2250+1.95% SP2401 with FPD operated in P-mode; held at 175°C for 2 min and increased to 240°C at 5°C/min and held 10 min. (1) Omethoate, (2) dimethoate, (3) chlorpyriphos, (4) azinphos-methyl, (a, b, c, and d) unidentified peaks, and (I.S.) parathion-methyl as internal standard.

ganophosphorus pesticides into 3 groups on the basis of polarity has been reported (3), but we did not adopt it because it worked poorly (i.e., incomplete separations, emulsion formation problems, and so on) and it is too time-consuming, requiring 9 partition equilibrations and 3 injections for each sample.

A further cleanup step has been included in the procedure because, even if the use of selective FPD detector minimizes interferences, repeated injections of samples with a large amount of coextractives are detrimental to GC columns, especially to a wide-bore column.

Cleanup is the most difficult step, especially for fatty foods: Florisil is a good adsorbent to eliminate fat, but this does not ensure a good recovery of the most polar organophosphorus insecticides (i.e., those insecticides with mercapto functionality, oxygenated analogs, and dimethoate).

In the literature, several methods for fatty extract cleanup have been proposed: gel permeation (3), a procedure based on low-temperature precipitation of fat (15), and disposable minicolumns of Kieselguhr-type material (5). We chose the latter procedure because in our preliminary tests, it proved very easy, fast, and efficient. Meat and eggs, however, require further cleanup on Supelclean LC18 (16).

To clean up nonfatty extracts from pigment coextractives, we used the Celite–carbon system (2, 4, 10, 14, 17), eluting with acetonitrile–benzene (1 + 1) (14).

Fractionation of pesticides by means of silica gel deactivated with 6.5% water (8, 9) can be useful for confirmation, and it proved essential prior to GC/MS analyses, because many extracts still had too high a load of coextractives for the capillary GC/MS system.

The SPB-5 0.53 mm id column with FPD detection was chosen as the main system for determining the organophospho-

rus residues in food, with the SP2250–SP2401 packed column as the confirmation column. In fact, the most frequent cases of closely eluting compounds on SPB-5 that we can meet in food samples (e.g., chlorpyriphos and parathion, chlorpyriphos-methyl and parathion-methyl, fenitrothion and pirimiphos-methyl) are well-resolved by SP2250–SP2401, as shown in Table 3. OV-101 was tested as well, but the elution pattern is essentially equivalent to that of SPB-5, thus limiting its usefulness.

The chromatograms of a bread extract and a lettuce extract on both columns are reported in Figures 2 and 3, respectively. There are no large interferences, and the comparison of SPB-5 and SP2250–SP2401 chromatograms allowed the peak identification.

Average results of recovery tests are to 81–85% for apples, whole milk, pasta, and eggs and 89% for olive oil. These results have been affected by the particularly low recoveries of demeton-*O* and demeton-*S*-methyl in the apples, whole milk, and olive oil and monocrotophos in the pasta, which are considered unacceptable.

Recoveries in the range 75–120% were considered acceptable, and the analytical results were not corrected for recovery. In the few cases with recoveries in the range 50–75%, the corresponding correction factor can be applied.

At the start of this study, our purpose was to determine also acephate and its metabolite methamidophos. The saturation of the aqueous layer with sodium chloride in the partition step (4, 12, 13) and the use of the carbon mixture without magnesium oxide (4) are 2 details included in the procedure to enhance acephate and methamidophos recovery (18). However, problems arose during the GC determination step, because acephate and methamidophos are easily degraded upon contact with the hot surfaces of the injection and/or column walls; cool on-column injection is probably the only suitable technique for these compounds, but it was considered impractical for this study.

The practical determination limit for the whole method according to the detection limits in Table 4 is in the range 3–50 ppb for a 25 g sample and a 1 μL injection out of a 1 mL final extract volume. In the case of oil, a 2.5 g aliquot was used, so the determination limit is 10 times higher.

Obviously, final extract and injection volumes can be modified. In particular, the injection volume can be increased up to 4 μL on wide-bore columns and to 10 μL on packed columns.

As the analytical characteristics—number of organophosphorus residues considered, ease of applicability (with slight modifications) to many vegetable and animal foods, and good determination limits—clearly show, the method reported here is suitable for a total diet study.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Enzyme-Linked Immunosorbent Assay for Quantitation of Organophosphate Pesticides: Fenitrothion, Chlorpyrifos-methyl, and Pirimiphos-methyl in Wheat Grain and Flour-Milling Fractions

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Simple, competitive enzyme-linked immunosorbent assays (ELISAs) have been developed for the quantitation of each of 3 major organophosphate insecticides: fenitrothion (FN), chlorpyrifos-methyl (CPM), and pirimiphos-methyl (PIRM). Performance of these assays on wheat grain and (for FN and CPM) on milling fractions such as flour, wheat germ, and bran has been assessed. Each assay is specific for the particular compound, i.e., no significant cross-reaction with the other 2 pesticides is observed. Only limited reactions were noted with major metabolites or analogs of these pesticides. Assay limits of detection of 0.3 ng FN, 0.2 ng CPM, and 0.02 ng PIRM, corresponding to limits of detection in whole grain of 0.08 ppm FN, 0.2 ppm CPM, and 0.03 ppm PIRM. Each compound in grain and milling fractions could be extracted quantitatively by simple shaking in neat methanol. Multiresidue analysis of the 3 insecticides was performed by simultaneously adding the cereal extract (diluted in phosphate buffer) to separate duplicate microwells coated with antibodies to FN, CPM, and PIRM and adding appropriate pesticide–horseradish peroxidase conjugates. High correlations between gas chromatography and the ELISA methods were obtained for insecticide levels in whole wheat and in milling fractions. In general, the ELISA assays had precision similar to those of instrumental pesticide analyses.

Most contracts for domestic or international sale of wheat grain or grain products such as flour specify a "nil" tolerance for live insect contamination (1). To meet this requirement in countries such as Australia, which store harvested grain for several months to years before domestic use or export, chemical insecticides are added to grain following harvest (1–5). The most commonly used grain protectants include organophosphates that have low mammalian toxicity, penetrate grain to a limited extent, and decompose slowly on storage. Residues of these organophosphates persist in grain and grain fractions (such as wheat germ, bran, white flour, and whole meal) destined for human consumption. Maximum residue limits in different products for each pesticide have been defined by the Codex Alimentarius Commission (6–8), but individual countries and particular companies commonly prescribe lower or "nil" residue limits. In the latter case, these are usually understood to be <0.1 ppm.

The current method of determining these organophosphate residues in wheat grain and grain products is gas chromatography (GC) with nitrogen-phosphorus or flame-photometric detection (2, 9–13), although some laboratories use liquid chromatography (13, 14) or thin-layer chromatography, sometimes associated with detection by cholinesterase enzyme inhibition (13, 15). Although these analyses are routine in the hands of skilled analysts in well-equipped laboratories, they are somewhat expensive if the objective is to screen out a few violating samples, and they are slow when large numbers of samples must be analyzed. Analysis of grain fractions may require cleanup prior to GC, and GC equipment is often not available in smaller or regional grain laboratories.

We have developed simple, quantitative enzyme-linked immunosorbent assays (ELISAs) for the 3 organophosphates most commonly used as grain protectants. Fenitrothion [*O,O*-dimethyl-*O*-(4-methyl-3-nitrophenyl) phosphorothioate, FN], chlorpyrifos-methyl [*O,O*-dimethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate, CPM], and pirimiphos-methyl [*O,O*-dimethyl-*O*-(2-diethylamino-6-methylpyrimidin-4-yl) phosphorothioate, PIRM] are used to different extents in Australia, Europe, Asia, and North America, in part because of different biological effectiveness against particular insect and mite species (5). Although PIRM is the most persistent of these organophosphates during storage and cooking, its mammalian toxicity is somewhat lower than the others (16). The 3 highly sensitive, direct, competitive ELISAs detected each compound individually over the low part-per-million

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Table 1. Cross-reaction of related pesticides in chlorpyrifos-methyl antibody test

Compound	Substituents					Cross reaction, %[a]
	R_1, R_2	R_3	R_4	R_5	X	
Analogs/related pesticides						
Chlorpyrifos-methyl	CH_3	Cl	Cl	Cl	N	100
Chlorpyrifos-ethyl	C_2H_5	Cl	Cl	Cl	N	15
Chlorpyrifos, monodechlorinated	C_2H_5	H	Cl	Cl	N	0.09
Chlorpyrifos, totally dechlorinated	C_2H_5	H	H	H	H	—[b]
Fenchlorphos	CH_3	Cl	Cl	Cl	C	40
Bromophos	CH_3	Cl	Br	Cl	C	60
Metabolites						
Chlorpyrifos-methyl oxon						1.0
3,5,6-trichloro-2-pyridinol						—[b]
Other agrochemicals						
Fenitrothion						3
Pirimiphos-methyl						—[b]
Parathion-methyl (*O,O*-dimethyl-*O*-4-nitrophenyl phosphorothioate)						0.8
Parathion (*O,O*-diethyl-*O*-4-nitrophenyl phosphorothioate)						
Dicapthon(*O*-2-chloro-4-nitrophenyl-*O,O*-dimethyl phosphorothioate)						3
Dichlofenthion [*O,O*-diethyl-*O*-(2,4-dichlorophenyl thiophosphate)]						0.2
Fenthion [*O,O*-dimethyl-*O*-(3-methyl-4-methylthiophenyl) phosphorothioate]						0.05
Tetrachlorvinphos [(*Z*)-2-chloro-1-(2,4,5-trichlorophenyl) ethenyldimethylphosphate]						—[b]
Triclopyr (3,5,6-trichloro-2-pyridyl oxyacetic acid)						—[b]

[a] CPM concentration yielding 50% inhibition × 100/(test compound concentration yielding 50% inhibition). Organophosphates showing <0.01% cross-reaction: azinphos-methyl, dichlorvos, dimethoate, temephos, malathion. Other pesticides showing <0.01% cross-reaction: bioresmethrin, phenothrin, permethrin, fenvalerate, methoprene, carbaryl.

[b] <0.05% or IC_{50} >100 μg/mL.

concentration ranges of importance in grain, without need for cleanup of extracts.

Experimental

Apparatus

(a) *Enzyme immunoassay reader.*—Bio-Rad Model 2550 (Bio-Rad, Richmond, CA 98404), or equivalent.

(b) *Microwell plates.*—Nunc Maxisorp polystyrene 96-well plates (Nunc, Roskilde, Denmark).

(c) *Shaker.*—IKA (Janke and Kunkel, Staufen, Germany), or equivalent.

Chemicals

(a) *Materials.*—Tween 20, 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), ovalbumin (OA), chicken IgG (IgY) (Sigma, St. Louis, MO 63178). Bovine serum albumin (BSA), horseradish peroxidase (HRP; Boehringher, Mannheim, Germany).

(b) *Solvents.*—Methanol, analytical grade (Ajax Chemicals, Clyde, NSW Australia).

(c) *Pesticide standards.*—FN and PIRM were obtained from Riedel-de-Hahn (Seelze, Germany) and CPM from Chem Service (West Chester, PA 19381). Other pesticides listed in Tables 1 and 2 were obtained from either Chem Service, Riedel-de-Hahn, or the Australian Government Curator of Standards, Melbourne, Victoria.

(d) *Protein-A or protein-G agarose.*—For antibody purification (Pharmacia, Uppsala, Sweden).

All other chemicals used were of reagent grade.

Reagents

(a) *Phosphate-buffered saline (PBS).*—0.15M NaCl–0.05M sodium phosphate, pH 7.2.

(b) *Potassium phosphate buffer.*—0.025M KH_2PO_4–0.15M NaCl–0.01% (w/v) NaN_3, pH 7.2.

Table 2. Cross-reaction of related pesticides in pirimiphos-methyl antibody test

Compound	Substituents			Cross-reaction, %[a]
	R_1, R_2	R_3	R_4	
Analogs/related pesticides				
Pirimiphos-methyl	CH_3	$N(C_2H_5)_2$	CH_3	100
Pirimiphos-ethyl	C_2H_5	$N(C_2H_5)_2$	CH_3	320
Diazinon	C_2H_5	$CH(CH_3)_2$	CH_3	0.7
Etrimfos	CH_3	C_2H_5	*O*-C_2H_5	0.02
Metabolite				
2-Diethylamino-4-hydroxy-6-methylpyrimidine				0.4
Other agrochemicals				
Fenitrothion				—[b]
Chlorpyrifos-methyl				—[b]
Chlorpyrifos-ethyl				—[b]

[a] Other compounds tested, by showing < 0.01% cross-reaction, are listed in Table 1. In addition, fenchlorphos, bromophos, parathion, parathion methyl, dicapthon, dichlofenthion, fenthion, tetrachlorvinphos, and triclopyr were nactive.
[b] < 0.05% or IC_{50} >100 μg/mL.

(c) *Coating buffer.*—0.05M sodium carbonate buffer, pH 9.6.

(d) *Washing buffer.*—0.05% (v/v) Tween 20 in PBS, pH 7.2.

(e) *Blocking solution.*—1% (w/v) BSA in PBS, pH 7.2.

(f) *Diluent buffer.*—0.05% (v/v) Tween 20–1% BSA in PBS, pH 7.2.

(g) *ABTS substrate.*—0.011% (w/v) ABTS in 0.1M sodium citrate buffer, pH 4.5, containing 0.003% (v/v) hydrogen peroxide.

(h) *Stopping reagent.*—3% (w/v) oxalic acid.

(i) *Pesticide stock solutions.*—10 mg FN, CPM, or PIRM in 10 mL methanol for preparation of dilutions for standard curve.

Preparation of Pesticide Conjugates

Conjugates of FN, CPM, and PIRM with both carrier proteins (for antibody production) or HRP (for use in the ELISA) were prepared by using a single spacer arm that incorporates one of the *O*-methoxy substituents and the phosphorothioyl ester moiety. Synthesis of the spacer arm, *O*-methyl-*N*-(*tert*-butylpropanoate)phosphoroaminothioyl chloride (I) has been described elsewhere (17).

FN–protein conjugates.—These were prepared using methods described elsewhere (18). The FN–succinimide ester was coupled to either IgY or HRP (17).

CPM–protein conjugates.—I (0.65 mmol) was refluxed overnight with sodium 3,4,6-trichloro-2-pyridyloxide (0.65 mmol) in 5 mL acetonitrile. Subsequent steps were as for the FN–protein conjugate preparation, except that the succinimidyl ester of the CPM derivative was purified by chromatography on silica gel eluted with ethyl acetate–petroleum ether (50 + 50) before coupling to proteins.

PIRM–protein conjugates.—2-Diethylamino-4-hydroxy-6-methylpyrimidine was deprotonated by reaction with sodium ethoxide, and the product (1 mmol) was refluxed with I (1.2 mmol) in 10 mL benzene overnight. Subsequent steps were as for the CPM–protein conjugate preparation, except that the succinimidyl ester of the PIRM derivative was purified by chromatography on silica gel eluted with chloroform. The identities of chemical intermediates in each synthesis were confirmed by proton NMR spectroscopy on 90-MHz (Joul FX90Q) or 200-MHz (Gemini 200) instrument. Infrared spectroscopy (Hitachi 279-30 spectrometer), elemental analysis, and melting-point determinations were also performed. Synthesis of these conjugates is described elsewhere (17).

Analysis of conjugates.—Conjugates were analyzed for protein content by using a colorimetric dye-binding assay (19), and the degree of hapten substitution with organophosphates was measured by determination of the loss of amino groups on the protein by using reaction with trinitrobenzenesulfonate (20). Conjugates for raising antibodies had the following hapten substitution ratios: (*1*) FN, coupled to IgY, 15 mol FN/mol IgY; (*2*) CPM, coupled to OA, 7.0 mol CPM/mol OA; (*3*) PIRM, coupled to OA, 7.2 mol PIRM/mol OA.

Antibody Production

Rabbits were immunized with PIRM–OA using the intradermal–intramuscular route (18), and monoclonal antibodies were prepared following immunization with FN–IgY or CPM–OA as previously described (18). Rabbit IgG antibodies were purified by protein-A agarose affinity chromatography; mouse monoclonal antibodies were purified by protein-G agarose affinity chromatography (21, 22). After dialysis against PBS, and concentration to 1–4 mg/mL, antibodies were stored at –20°C.

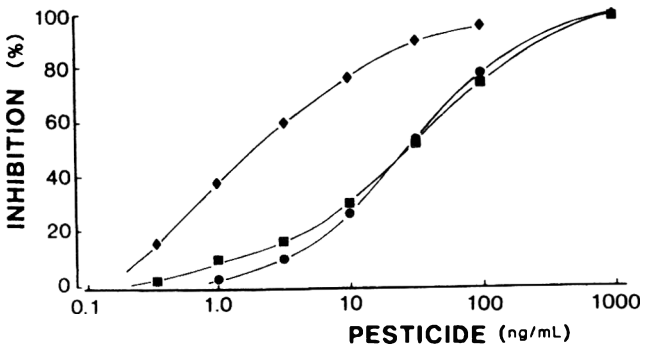

Figure 1. Standard curves for analysis of (●) FN, using FN-specific antibody; (■) CPM, using CPM antibody; (◆) PIRM, using PIRM antibody. Data are means of 5–10 assays.

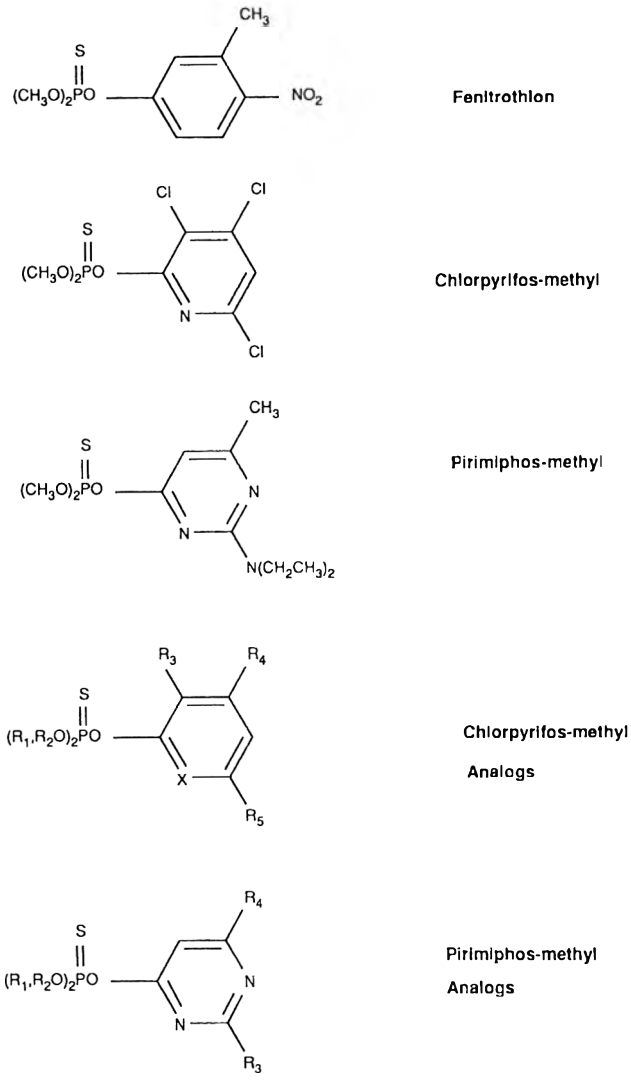

Figure 2. Structures of FN, CPM, and PIRM, and keys for structures of analogs and metabolites of CPM and PIRM.

Organophosphate Assays

Sample preparation.—Wheat grain samples were obtained from commercially treated bulk storages 2–18 months after treatment. Pesticide was extracted by standing 10 g grain in 25 mL methanol for 45–48 h in stoppered 100 mL flasks at 20°C. Flasks were shaken at 200 rpm for 15 min twice during the extraction period. This method has been shown to be optimal for quantitative extraction of organophosphates from wheat grain (1, 13).

Milling fractions were obtained from commercial roller milling as part of the Australian Grain Protectants Working Group trials. Flour and germ were extracted by using 10 g/50 mL methanol, and bran was extracted by using 5 g/50 mL methanol.

FN assay.—Monoclonal antibody PF10/20 was diluted to 10 μg/mL with coating buffer, and 100 μL was added to each well of a 96-well plate and incubated 1 h at 20°C. After washing all wells 3 times with 250 μL PBS–Tween to remove unbound antibody, 150 μL blocking solution was added, and the wells were incubated 1 h at 20°C. Standards, methanol (for controls and blanks), and methanol grain extracts were routinely diluted 1:25 with diluent buffer. For grain samples containing <1 ppm FN, methanol extracts were diluted 1:5 with diluent buffer. After removal of blocking solution, 50 μL diluted methanol was added to control wells, and diluted methanol grain extract or diluted FN standard was added appropriately to separate wells in triplicate. To each well, 50 μL FN–HRP conjugate (2.5 ng enzyme) in diluent buffer was immediately added. Well contents were mixed by gentle agitation of the microplate for 10 s. After 30 min incubation at 20°C, the plate was washed 3 times, and 150 μL substrate–chromogen was added. The mixture was incubated 15 min at 20°C. To each well, 50 μL stopping reagent was added, and absorbance was measured at 414 nm.

CPM assay.—Monoclonal antibody PCP7/2 was used. The assay was performed as for the FN assay, except that methanol controls, grain extracts, and CPM standards were diluted 1:20 with diluent buffer. In each well, 20 ng CPM–HRP was used. For analysis of methanol extracts of flours, bran, and germ, extracts were first diluted by addition of an equal volume of methanol and then further diluted 1:10 with diluent buffer.

PIRM assay.—A polyclonal antibody was used. The assay was performed as for the FN assay, except that PIRM standards were used. The amount of PIRM–HRP used per well was 40 ng.

GC analyses.—Grain samples and milling fractions were extracted using methanol as described above. The organophosphate residues were determined by the Australian Wheat Board

(a National Association of Testing Authorities, Australia, accredited laboratory), using GC with flame photometric detection in the phosphorus mode (13).

Data Analysis

Data points for standards were fitted using software (Microman, Bio-Rad) that provided a 4-parameter logistic plot. Alternatively, reasonably accurate estimates of pesticide in test samples may be obtained by use of a log(concentration) vs absorbance plot, providing that only the central region (25–75% maximum absorbance) of the plot is used for determinations. To compare the GC and ELISA methods, computer-assisted linear regression analyses were performed.

Results and Discussion

Assay Optimization

Standard curves for the FN, CPM, and PIRM assays are shown in Figure 1. Structures of these pesticides are shown in Figure 2. Data are expressed as percent inhibition of absorbance obtained for controls when assays are performed in the presence of 5% methanol but in the absence of competing pesticide. In each case, the control absorbance (optical density) values (A_{414}) were 0.8–1.2. Data shown are means of standard curves performed on 4–10 separate days. The assays used a polyclonal antibody to PIRM and monoclonal antibodies to FN and CPM, but had roughly similar detection sensitivity and dynamic response. The assay for PIRM was more sensitive than the FN and CPM assays, with 50% inhibition of antibody binding (IC_{50}) at 0.21 ng PIRM, and a limit of detection (10% inhibition) of about 0.02 ng. The corresponding IC_{50} values for FN and CPM were 2.8 ng and 2.9 ng, respectively, with sensitivities of 0.3 ng and 0.1 ng, respectively. The requirement for a slightly more sensitive assay for PIRM is in keeping with the lower application rates used for PIRM.

The slopes of the 3 plots were similar, with the FN curve being a little more dynamic (i.e., steeper). A 5-fold difference in pesticide concentration over the steepest part of the organophosphate concentration vs antibody-binding standard curves gave the following differences in inhibition: FN, 36%; CPM, 29%; and PIRM, 35%. Thus, the CPM standard curve was slightly less steep than the FN or PIRM curves. The particular antibodies and assay formats for individual assay of the 3 pesticides were selected on the basis of *(1)* appropriate sensitivity for the pesticide, *(2)* steep concentration-response characteristics (enabling discrimination of samples differing by no more than 20% in pesticide), *(3)* simplicity of use, and *(4)* freedom from vehicle and matrix effects. A variety of assay formats (e.g., pesticide–protein complex or antibody bound to the microwell) and antibodies (polyclonal antisera to protein–organophosphate conjugates prepared at different molar hapten:protein ratios or different monoclonal antibodies) exist (18), but the formats chosen were most suited, especially on the basis of producing simple assays over overlapping concentration ranges for the 3 compounds. Other antibodies and formats enabled somewhat more sensitive detection of pesticide; for example, a polyclonal antibody was developed that was able to detect <0.01 ppb CPM (Skerritt, Hill, and Edward, unpublished data). However, although such sensitivity may be of use in environmental monitoring applications, the need to dilute extracts of grain samples over 1000-fold before analysis with this antibody would introduce some loss of analytical precision and accuracy; therefore, this antibody is not favored for grain pesticide analysis.

Antibody Specificity

The antibodies were analyzed for reaction with other pesticides and with major metabolites of the particular pesticide under study. These data are of some importance because *(1)* a variety of legal (approved) or illegal pesticides can be encountered on stored grain; *(2)* organophosphates are often used in combination with other pesticides, such as pyrethroids, to enable control of a broad range of insect pests; and *(3)* significant amounts of pesticide metabolites can accumulate in grain during long-term storage (1, 23).

Specificity properties of the FN antibody used in the test have been described elsewhere (18); those for CPM and PIRM are shown in Tables 1 and 2. Briefly, the FN assay did not recognize CPM, PIRM, or other grain protectants such as bioresmethrin, (*R*)-1-phenothrin, dichlorvos, carbaryl, malathion, or methoprene. Some structurally related organophosphates (e.g., parathion, parathion-methyl, and dicapthon) were recognized, but rather weakly; their IC_{50} values were 50–300 times greater than that of FN.

The CPM assay was functionally specific to a similar degree (Table 1), not detecting many other organophosphates or pesticides from other chemical groups. A number of agrochemicals are known with a single di-or trichlorinated pyridine or benzene ring. These were assessed in the assay. Of these, chlorpyrifos-ethyl (not used on grain) and fenchlorphos, the benzene analog of CPM (now very rarely used on grain), were 15–60% as active as CPM with the monoclonal antibody used in this assay, although with other antibodies to CPM, fenchlorphos was only very weakly detected (Edward, Hill, and Skerritt, unpublished data). A bromo-analog of fenchlorphos—bromophos (which also reacted in the assay)—has been used occasionally as a grain protectant (1). The major metabolite of CPM—3,5,6-trichloro-2-pyridinol—did not react appreciably with the antibody (<0.01% cross-reaction, IC_{50} = 900 μg/mL).

The only major grain protectant that cross-reacted weakly in the CPM assay was FN (3% cross-reaction). Very high amounts (15 ppm in grain) would be required before it would be detected in this assay. The polyclonal antibodies to CPM did not recognize FN. It can be concluded that the CPM monoclonal antibody can recognize either substituted pyridinol or phenol-type organothiophosphates with substituents on one or more of the positions *ortho-* or *meta-* to the phosphate ester. Although substitution of the methyl groups for ethyl groups reduced antibody binding 6-fold, alteration to the phosphate ester abolished binding.

Tetrachlorvinphos and trichopyr had 3 chlorine atoms substituted at appropriate positions on the aromatic ring, but were inactive. However, tetrachlorvinphos (an analog of dichlorvos)

Table 3. Matrix effects in organophosphate ELISAs[a]

	Percentage inhibition of antibody binding									
	Fenitrothion				Chlorpyrifos-methyl				Pirimiphos-methyl	
Pesticide, ng/mL	Vehicle	Grain	Flour	Bran + Germ	Vehicle	Grain	Flour	Bran + Germ	Vehicle	Grain
1.0	0	0	0	0	5	6	8	7	11	11
3	12	11	13	10	12	10	13	11	29	27
10	18	23	21	18	23	20	20	22	49	47
30	41	44	41	38	49	46	49	47	71	69
100	70	74	66	65	67	62	67	61	86	85
1000	96	97	95	94	96	94	94	93	98	98

[a] Data shown are from a typical experiment, which was replicated 2–3 times with similar results.

had a substituted methylene group between the phosphate ester and the aromatic ring, and trichlopyr (a herbicide) is a substituted oxyacetic acid, being the pyridine analog of 2,4,5-T.

The PIRM assay (Table 2) did not detect FN, CPM, or any other organophosphate tested, except pirimiphos-ethyl, which is not used on grain. Etrimfos, used as a grain protectant in the United Kingdom (17), and diazinon, a widely used organophosphate, are 2,6-substituted analogs of PIRM and pirimiphos-ethyl, respectively, but were only very weakly detected.

In summary, these cross-reactivity data indicate that false-positive results for the 3 organophosphates should not arise with the currently available grain protectants over the concentration ranges that these compounds would normally be applied. As indicated in our earlier publication (18), FN metabolites present in highly aged grain samples (i.e., treated with FN some years earlier) may show a slight reaction in the FN assay. However, such samples would be analyzed rarely.

Matrix Effects

Different concentrations of each pesticide prepared in a methanol stock solution were spiked into either neat methanol or methanol extracts of pesticide-free whole-wheat grain, flour, or a bran–germ mixture, allowed to stand 1 h at 20°C and then diluted appropriately for the assay. Concentration-inhibition data for each pesticide prepared in methanol or in the 3 methanol cereal extracts were superimposable, indicating the absence of matrix effects (Table 3).

Precision of the Assay

The intra-assay repeatability and between-assay reproducibility of concentration-inhibition curves for each of the 3 pesticides were studied (Table 4). The standard curves were very reproducible, as indicated by low coefficients of variation.

Precision results were in keeping for determinations in the low- to subnanogram range (24). However, standard curves for the compound or compounds under study should be performed simultaneously with analysis of unknowns.

Part of the error in the reproducibility data is also likely to be due to day-to-day variation in the preparation of fresh pesticide stocks. Preparation of fresh stocks (100 μg/mL) has subsequently been found not to be necessary, provided that the methanol solutions of pesticide are stored below 25°C with the addition of 1% acetic acid to the stock, which did not affect the assay (Desmarchelier, Skerritt, Hill, and Beasley, unpublished

Table 4. Precision of organophosphate ELISA standard curves[a]

	Percentage inhibition of antibody binding											
	Fenitrothion				Chlorpyrifos-methyl				Pirimiphos-methyl			
Pesticide, ng/mL	Within-assay mean (n = 3)	SD	Between-assay mean (n = 10)	SD	Within-assay mean (n = 3)	SD	Between-assay mean (n = 8)	SD	Within-assay mean (n = 5)	SD	Between-assay mean (n = 4)	SD
0.3	—	—	—	—	—	—	—	—	15	3.7	15	4.2
1.0	0.1	—	0.3	0.1	6.2	1.4	9	1.3	35	3.3	37	4.9
3	12	1.4	9	0.3	13	1.1	15	2.6	49	3.4	58	10.2
10	22	2.4	26	5.2	24	2.7	29	3.2	69	2.4	75	5.9
30	51	5.6	53	6.8	50	0.6	51	3.4	84	2.5	88	2.4
100	74	2.5	76	3.9	68	0.7	72	2.2	93	1.6	93	1.6
1000	96	1.0	97	1.0	96	0.4	94	0.4	99	1.0	96	3.6

[a] n = number of assays.

Table 5. Precision of organophosphate ELISAs vs GC for low-, medium-, and high-pesticide wheat samples[a]

	Assay								
	Fenitrothion			Chlorpyrifos-methyl			Pirimiphos-methyl		
	ELISA			ELISA			ELISA		
Pesticide level	Mean ± SD	CV, %	GC value	Mean ± SD	CV, %	GC value	Mean ± SD	CV, %	GC value
Low	1.8 ± 0.28	15	1.7	2.0 ± 0.48	24	2.3	2.3 ± 0.25	12	2.4
Medium	3.3 ± 0.36	11	3.0	5.7 ± 0.63	11	5.0	6.7 ± 0.68	12	6.4
High	5.3 ± 0.47	9	5.8	9.1 ± 2.07	23	7.6	10.3 ± 1.0	10	9.5

[a] ELISA data shown are for 5 assays performed sequentially on separate days; GC values are from single analyses.

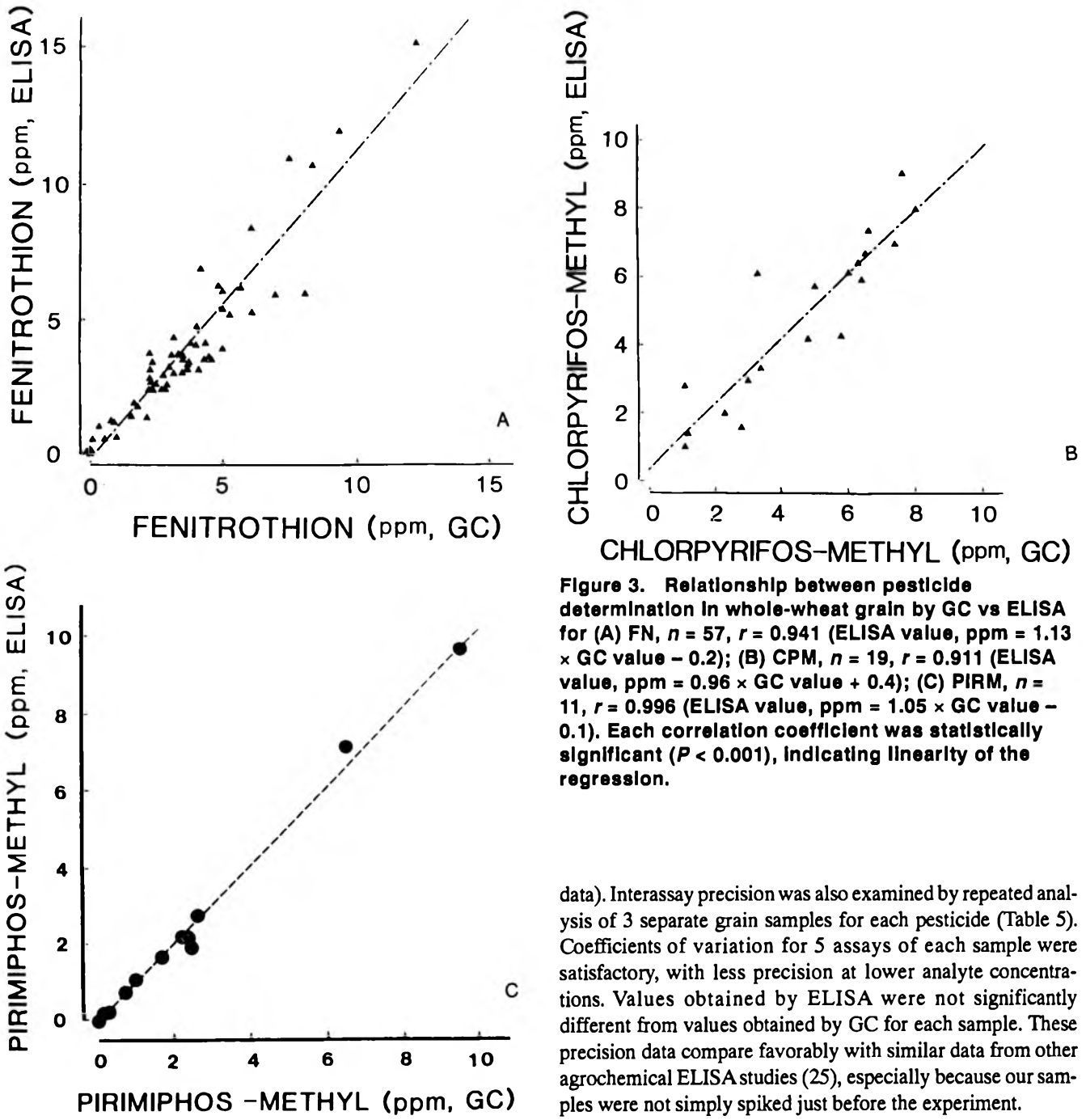

Figure 3. Relationship between pesticide determination in whole-wheat grain by GC vs ELISA for (A) FN, $n = 57$, $r = 0.941$ (ELISA value, ppm = 1.13 × GC value − 0.2); (B) CPM, $n = 19$, $r = 0.911$ (ELISA value, ppm = 0.96 × GC value + 0.4); (C) PIRM, $n = 11$, $r = 0.996$ (ELISA value, ppm = 1.05 × GC value − 0.1). Each correlation coefficient was statistically significant ($P < 0.001$), indicating linearity of the regression.

data). Interassay precision was also examined by repeated analysis of 3 separate grain samples for each pesticide (Table 5). Coefficients of variation for 5 assays of each sample were satisfactory, with less precision at lower analyte concentrations. Values obtained by ELISA were not significantly different from values obtained by GC for each sample. These precision data compare favorably with similar data from other agrochemical ELISA studies (25), especially because our samples were not simply spiked just before the experiment.

Accuracy of Results Obtained from Whole-Wheat Grain

In these studies, ELISA data obtained from individual wheat samples were compared with GC data obtained from single analyses of the same samples. Excellent correlations between the ELISA and the GC data were obtained for each organophosphate (Figure 3), and in each case, the regression coefficients were highly statistically significant, indicating linearity. The regression lines had slopes very close to 1 and intercepts near the origin.

The correlation between ELISA and GC values for CPM was slightly inferior (Figure 3B). This is related to the instability of CPM to hydrolysis both on grain and in solution. Following the GC analyses and prior to ELISA analyses, slight CPM degradation in some samples may have occurred. Also, the enzyme-labeled reagents used in the ELISA were somewhat less stable than the corresponding FN and PIRM reagents. We have since obtained excellent stability by using a stable analog of CPM coupled to HRP in the assay (McAdam, Hill, Edward, and Skerritt, unpublished data). The CPM assay was also less sensitive than the others. Extracts of grain required higher dilution prior to analysis, because of sensitivity of the antibody to methanol. Thus, only samples containing more than 1 ppm CPM were analyzed.

Accuracy of Results Obtained with Flour, Bran, and Germ Samples

Milling fractions were obtained from commercial trials with grain treated with either FN or CPM. Close correlations between GC and ELISA data were seen for FN in flour samples, which had residues in the range 0.7–6.1 ppm (Figure 4A). The range of pesticide concentrations in flour, bran, and germ samples (determined by GC) for CPM (Figure 4B) was somewhat higher than that for FN. However, the aqueous solubility of CPM is 4-fold lower than that for FN (26), and the CPM concentration in diluted extracts of flour, bran, and germ samples would approach its limit of aqueous solubility. For this reason, data obtained by direct 20-fold dilution in buffer of the CPM-containing extracts of wheat and the milling fractions were compared with data obtained by first diluting the extract 2-fold in methanol and then 10-fold in buffer. There was not a significant difference between the results obtained using either method with whole-wheat samples, because CPM levels in these were low.

With FN, the slope of the GC vs ELISA data plot was near unity for brans and germ, when extracts were diluted directly in buffer (Figure 5A). However, in flours (Figure 4B) and bran and germ samples (Figure 5B), correlations between ELISA and GC data for CPM were better when the latter method was used. For flours, ELISA value = 0.87 × GC value + 0.73 (r = 0.974), when an intermediate methanol dilution was performed, and ELISA value = 0.82 × GC value + 1.1 (r = 0.869), for direct dilution into buffer. For brans and germ samples, ELISA value = 0.83 × GC + 2.9 (r = 0.757), using methanol predilution and ELISA value = 0.77 × GC + 5.1 (r = 0.699), when extracts were diluted directly into buffer. The intercept for the CPM data plot for the set of bran and germ samples we analyzed probably did not pass through the origin because the set did not have samples containing less than about 15 ppm CPM. Further studies using germ and bran samples containing lower levels of CPM will be necessary to validate the method in the 0.5 to 15 ppm range for CPM. Nevertheless, direct dilution of extracts into buffer is slightly sim-

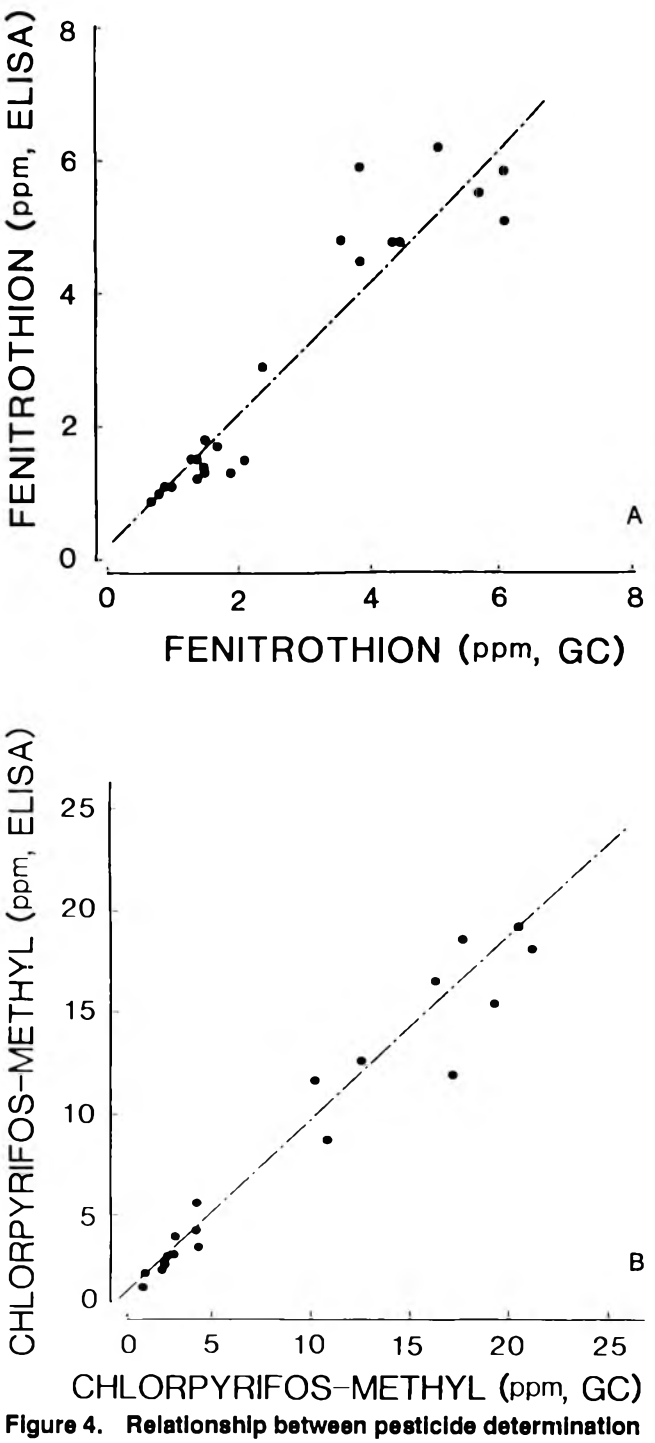

Figure 4. Relationship between pesticide determination in flours by GC vs ELISA for (A) FN, n = 23, r = 0.947 (ELISA value, ppm = 1.02 × GC value + 0.1); (B) CPM, n = 19, r = 0.974 (ELISA value, ppm = 0.87 × GC value + 0.7). Each correlation coefficient was statistically significant (P < 0.001), indicating linearity of the regression.

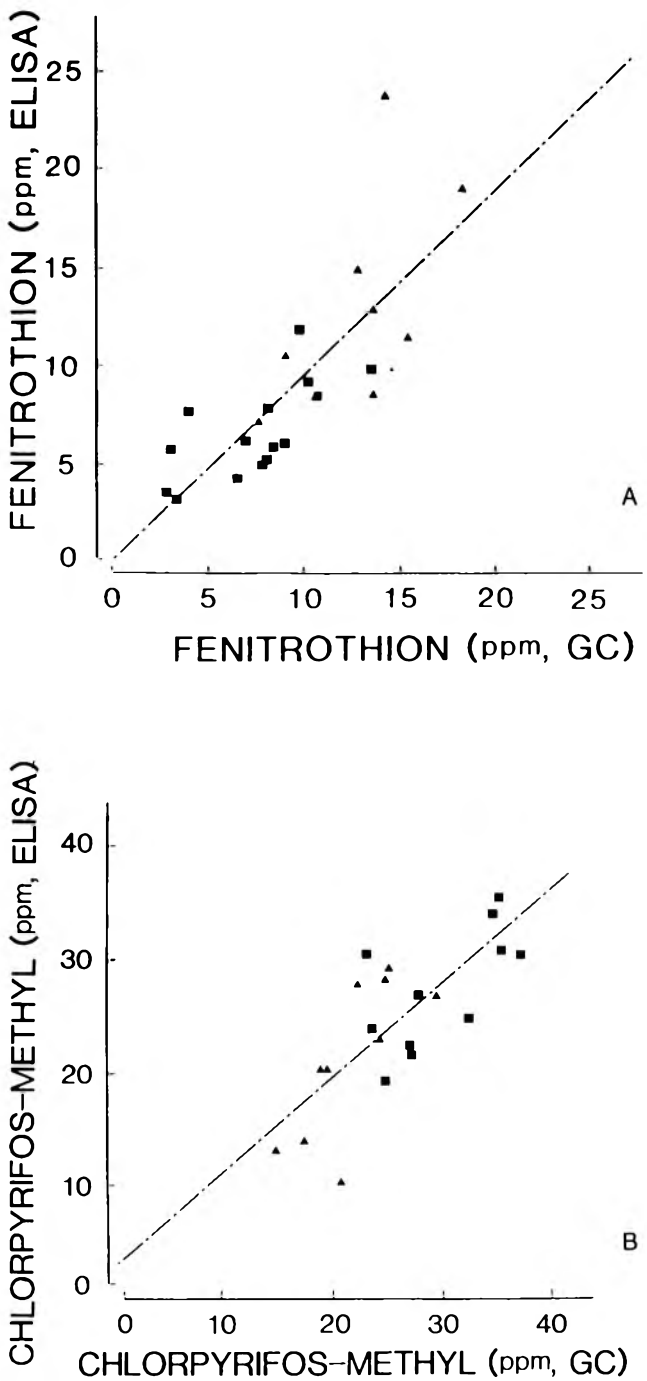

Figure 5. Relationship between pesticide determination in wheat germ ▲ and bran ■ fractions by GC vs ELISA for (A) FN, $n = 23$, $r = 0.710$ (ELISA value, ppm = 0.97 × GC value − 0.1); (B) CPM, $n = 21$, $r = 0.757$ (ELISA value, ppm = 0.83 × GC value + 2.9). Each correlation coefficient was statistically significant ($P < 0.001$), indicating linearity of the regression.

pler and should suffice where semiquantitative analyses are appropriate. Although germ is a lipid-rich fraction, cleanup of samples was not necessary.

Data for bran and germ are considered together, first, because in several milling operations the fractions are pooled as mill "offal," and second, because clustering of pesticide analytical values in the bran samples available would have made it difficult to establish meaningful separate regressions.

Conclusion

Immunoassays have been developed for the major organophosphates used on grain in storage and have been applied to the quantitative determination of these compounds in wheat and flour milling fractions. This contrasts with many other pesticide ELISAs, which have been designed to be very sensitive but not necessarily quantitative (27–29). The FN and PIRM methods were, in general, as accurate and precise as GC methods, which have shown intralaboratory coefficients of variation of 4–15% and interlaboratory imprecision of 15–25% (Flour Millers' Council of Australia, unpublished data; 2, 30). Another source of intra-assay variation common to both ELISA and GC analyses results from sampling variation; pesticide within a bulk wheat sample is not evenly distributed among different 10–100 g subsamples.

The CPM method was only a little less precise, and with improvements in reagent stability, assay precision has been enhanced. GC determinations are typically based on sequential analyses requiring 15–30 min each. The ELISA methods should have the advantages of higher throughput and lower expense, because large numbers of samples (up to 100) can be analyzed simultaneously in a 60 min assay, by using microwells that had been precoated with antibody. The methods can be applied to the quantitative screening of residue levels in large numbers of grain samples. Samples that appear to violate official Maximum Residue Levels would be subjected to a confirmatory analysis by GC. The ELISA methods can also be used to confirm whether sufficient pesticide has been applied and whether application is even throughout a stored grain bulk. A final application is segregation of grain for "pesticide-free" markets. Following assessment of matrix effects and validation studies, the assays are potentially adaptable to other matrixes such as baked goods, vegetables, and citrus fruits, as well as to environmental samples, where these organophosphates may be used.

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PLANT TOXINS

Analysis of 5-Vinyl-1,3-Oxazolidine-2-Thione by Liquid Chromatography

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5-Vinyl-1,3-oxazolidine-2-thione (5-VOT) is a goitrigenic compound released by enzymatic degradation of progoitrin, the major glucosinolate occurring in rapeseed meal. A liquid chromatographic (LC) method for determination of 5-VOT in a biological environment is presented. Complete extraction of 5-VOT has been carried out by complexation with phenyl mercury acetate under cyclohexanic conditions, and then by decomplexation using an aqueous sodium thiosulfate solution. These reactions displace 5-VOT from an aqueous to an organic medium, and then back again to the aqueous condition, thus assuring high selectivity of the extraction. Precise quantitation of 5-VOT is completed in 10 min by reversed-phase liquid chromatography using an isocratic elution with UV detection and a specially made synthetic internal standard. Concentration steps by solid-phase chromatography and evaporation can be introduced in the analytic procedure to lower the detection limit of 5-VOT in the sample used from 100 to 0.5 ppb. Using sow milk samples, the method was tested by small measured additions of 5-VOT. The recovery rate of the product was very good (>97%). Different phases used to achieve a sensitive, rapid, and precise method are described.

Rapeseed meals are being used more and more frequently in animal feed because of the lower glucosinolate content in newer varieties. Present in the seeds, these compounds remain in the meals after oil extraction and are easily broken down by endogenous myrosinase, or in vivo by the bacterial flora in animals. Degradation products, especially 5-VOT stemming from the 2-hydroxybut-3-enyl glucosinolate (progoitrin), produce antinutritional effects (Figure 1). In addition, 5-VOT inhibits the synthesis of thyroid hormones, which, in turn, causes metabolic disturbances (1). Examples of this can be seen in hogs yielding less meat or in fowl having lower fertility (2) with eggs giving off a fishy odor. Moreover, 5-VOT is transferred to serum, milk, and muscular tissues, and to certain organs such as the liver, kidney, and thyroid. Consequently, its indirect consumption presents a potential danger not only to animals but also to humans.

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The amount of 5-VOT in feed must be controlled to guarantee quality animal breeding. Experiments performed on ruminants showed that varying levels of 5-VOT were found in biological fluids and tissues: from 3 to 8 ppb in milk, 15 to 200 ppb in plasma, 80 to 250 ppb in urine (3), 70 ppb in thyroid, and less than 0.5 ppb in liver or kidney (4). A very sensitive method appears necessary for investigating such samples. On the basis of work done by Astwood et al. (5), Kreula et al. (6) were the first to analyze 5-VOT in complex environments. First, 5-VOT found in plant extracts or milk was isolated by 2-dimensional paper chromatography, and then quantitated by UV spectrometry. Some researchers then used gas chromatography (GC), but detection limits were insufficient for precise analyses in a physiological medium (7, 8). In 1979, MacLeod et al. (9) introduced liquid chromatography to the investigation. Before analysis using gas chromatography with detection by electron capture of the butylheptafluoro derivative, liquid chromatography was used to extract 5-VOT. The same year, Josefsson et al. (10) and Benns et al. (11) quantitated 5-VOT obtained from liquid extraction directly by normal-phase polarity liquid chromatography. In 1982, De Brabander and Verbeke extracted 5-VOT in a very selective manner by complexation with a mercuric compound (4, 12), after gas chromatography with electron capture detection of the derivative formed by pentafluorobenzoyl chloride. The detection limit was, thus, lowered to less than 1 ppb (3).

In this paper, we describe a method based on De Brabander's selective extraction principle with the same sensitivity and selectivity, but by isocratic liquid chromatography and UV detection. In the present case, this technique is advantageous in that it is a commonly used technique in laboratories, does not require derivation reagents, needs less analysis time, and may be automated.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Model 64 pump (Knauer, Berlin, Germany) equipped with Model 7125 injection valve

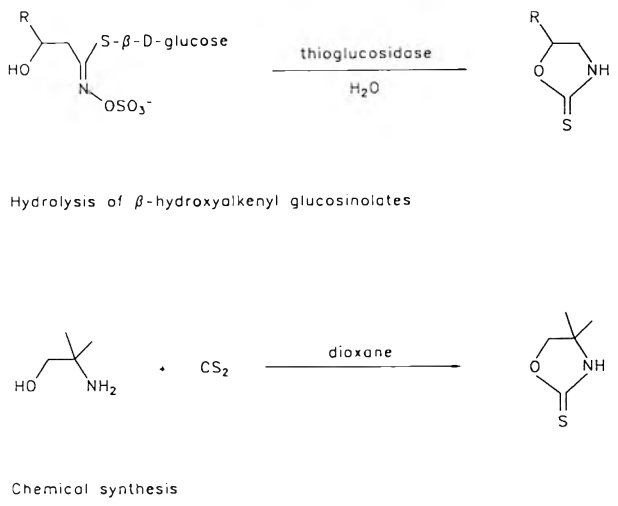

Figure 1. Natural (a) and synthetic (b) formation of oxazolidine-2-thiones. (a) R: CH_2=CH- : 2-hydroxybut-3-enyl glucosinolate (progoitrin) is hydrolyzed into 5-vinyl-1,3-oxazolidine-2-thione (5-VOT or goitrin). R: CH_2=CH-CH_2- : 2-hydroxypent-4-enyl glucosinolate (gluconapoleiferin) is hydrolyzed into 5-allyl-1,3-oxazolidine-2-thione (5-AOT). (b) Condensation of 2-amino-2-methylpropan-1-ol and carbon disulfide in dioxane yields 4,4-dimethyl-1,3-oxazolidine-2-thione (4,4-DMOT)(internal standard for LC analysis).

with 20 µL loop (Rheodyne, Inc., Cotati, CA 94928). Model 87 spectrophotometric detector with variable UV wavelength (Knauer). Scanning UV detector (Model Focus, Spectra Physics, San Jose, CA 95134). Lichrospher C8 chromatographic column 5 µm, 125 × 4 mm with Manucart connectors (E. Merck, Darmstadt, Germany). Model C-R3A recorder/integrator (Shimadzu Corp., Kyoto, Japan).

(b) *Stirrer.*—Dangoumau-type with alternative action (frequency: 350 counts/min; amplitude: 40 mm).

(c) *Solid-phase chromatography system.*—Elution aspirating device (Model 10-SPE, Baker, Deventer, The Netherlands), cartridges packed with 200 mg 40 µm C18 bonded silica (Baker).

(d) *Evaporator.*—Reacti Vap evaporator (Pierce Chemical Co., Rockford, IL 61105).

(e) *Centrifuge.*—5000 t/min MLW T5 (Bioblock, Illkirch, France). 100 × 16 mm glass tubes with Teflon-faced screw caps.

Reagents

(a) *For 5-VOT extraction.*—1% phenolphthalein solution in ethanol (E. Merck); 5% sodium hydroxide solution in water; saturated phenyl mercury acetate (PMA) in cyclohexane prepared as follows: heat ca 100 mg PMA (E. Merck) and 150 mL cyclohexane (E. Merck) in 250 mL flask at reflux for 30 min, cool, and then pass solution through 0.22 µm Teflon filter, and store in darkness at room temperature. 0.1M Sodium thiosulfate solution in water (E. Merck).

Table 1. Low detection limits[a] (ppb) of method according to sample weight and volume injected in liquid chromatograph

	Sample weight, g		
Injected volume, µL	0.2	0.5	1.0
20	5.0	2.0	1.0
50	2.0	0.8	0.4

[a] Ratio signal/noise (S/N) = 4 and 50% OT lost during sample preparation.

(b) *For extract purification by solid-phase chromatography.*—Methanol (SDS, Peypin, France) and 70% (v/v) acetonitrile in water (E. Merck).

(c) *For LC analysis.*—10% (v/v) acetonitrile in water (E. Merck).

(d) *For standard solutions.*—5-vinyl-1,3-oxazolidine-2-thione in water (National Physical Laboratory, Teddington, UK) at 100 ppm in water. 4,4-Dimethyl-1,3-oxazolidine-2-thione (4,4-DMOT) at 100 ppm in water. These solutions are diluted with respect to samples to be analyzed. 4,4-DMOT, internal standard, is synthesized by condensation of CS_2 on 2-amino-2-methyl-1-propanol in dioxane (13).

Sample Preparation

(a) *5-VOT extraction by complexation/decomplexation.*—In glass tube (100 × 16 mm), dissolve 200 mg lyophilized substance to be analyzed in 3 mL water. Add same quantity of internal standard (4,4-DMOT) in solution in water as 5-VOT presumably contained in sample. Close tube and shake vigorously. After adding drop of phenolphthalein solution, adjust to basic pH (rose colored) with sodium hydroxide solution. Add 3 mL saturated PMA solution to tube, place in stirrer for 2 min, and centrifuge 5 min at 5000 t/min. Transfer cyclohexanic phase to another tube (100 × 16 mm) and add 3 mL 0.1M sodium thiosulfate solution. After vigorous manual shaking (15 sec) and centrifuging (5 min at 5000 t/min), remove aqueous phase. It is now ready for LC analysis if 5-VOT concentration is greater than 500 ppb. If this is not the case, sodium thiosulfate must be eliminated and 5-VOT concentrated.

(b) *Sodium thiosulfate elimination and 5-VOT concentration.*—Wash cartridge containing C18 bonded silica (200 mg) with 2 mL methanol and 10 mL water, taking care to prevent any air from entering. Apply solution to be purified (ca 2.5 mL) to cartridge reservoir, and then percolate. Wash C18 phase with 1 mL water to eliminate thiosulfate, and then carry out 5-VOT elution with 0.5 mL 70% (v/v) acetonitrile in water. Eluate is then ready for LC analysis if amount of 5-VOT in original sample is greater than 50 ppb. If this is not the case, solution must be concentrated to 50 µL by evaporating under nitrogen current. By injecting 20 µL of this concentrated solution into LC column, detection limit of 5 ppb is reached (S/N = 4). Lower detection limit may be achieved by increasing both volume injected (50 µL) and sample weight (from 0.2 g to 1.0 g) (Table 1).

(c) *Analysis by reversed-phase LC.*—Elute in isocratic mode with 10% (v/v) acetonitrile in water at flow rate of 1 mL/min at room temperature. Under these conditions, retention times for 4,4-DMOT, 5-VOT, and 5-AOT are 4.3, 4.9, and 9.3 min, respectively (Figure 2). UV detection is performed at 241 nm or 254 nm when using fixed wavelength UV detector.

Results and Discussion

5-VOT Characteristics

5-VOT, an intermediary polar compound, is stable in a basic medium and has a pKa equal to 10.5. Table 2 shows distribution coefficients of 5-VOT between different organic solvents and aqueous solutions with acid, basic, or neutral pH. Values obtained by De Brabander et al. are more easily explained with the help of the polarity and proton-acceptor parameters (14). With an acid pH (pH = 1), the solubility of 5-VOT is increased with the polarity of the organic solvent; however, the proton-acceptor character of ethyl acetate and ethyl ether greatly diminishes their solvent power. With a neutral pH (pH = 7), the solubility increases with the polarity of the organic solvents. With a strong basic pH (pH = 13), 5-VOT is ionized and becomes practically nonextractable by the organic solvents.

The analysis of 5-VOT in reversed- or normal-phase polarity liquid chromatography provides evidence for and completes these observations. Retentions of 5-VOT were measured following the systems described in Tables 3 and 4. k' capacity factors show that greater retentions were obtained with a pure aqueous eluant in phenyl- and octyl-bonded phases, or with a nonaqueous eluant in hexane in all stationary phases. Thus, liquid chromatography can analyze 5-VOT obtained from either an aqueous or organic phase after extraction. With respect to the extraction mode, the nature of the intermediary polarity of 5-VOT indicates that a very selective liquid-liquid extraction would be difficult to carry out. Indeed, 5-VOT would not be separated from numerous impurities of a neighboring polarity

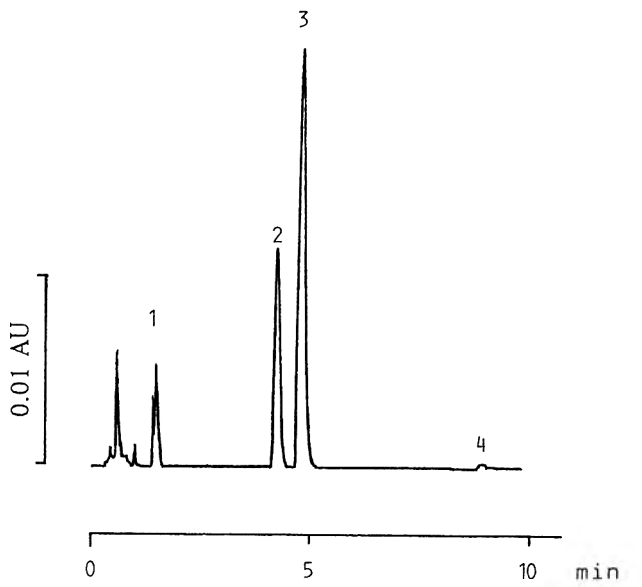

Figure 2. Chromatogram of sow milk sample prepared according to method described (see *Experimental*). Peak identification: 1: $Na_2S_2O_3$; 2: 4,4-DMOT (internal standard); 3: 5-VOT; 4: 5-AOT. Internal standard amount: 5 μg/g lyophilized milk.

found in physiological fluid samples. Moreover, 5-VOT can be linked to the biological matrix by bonds strong enough to make the quantitative extraction incomplete.

5-VOT Extraction

(a) *Complexation.*—To improve selectivity and yields from the extraction of 5-VOT in an aqueous medium, phenyl mercury acetate was used. This compound is well-known for its capacity to form specific complexes with thyreostatic molecules (4, 12). A cyclohexanic solution saturated in PMA extracted 5-VOT from an aqueous solution at basic pH by forming a complex only soluble in the organic phase. The ex-

Table 2. Distribution coefficients of 5-VOT between aqueous solutions at acid, neutral, and basic pH, and different organic solvents

	Solubility parameters		Distribution coefficients[a] Aqueous phases		
Solvent	Polarity[b]	PASP[c]	1.0 pH	7.0 pH	13.0 pH
Hexane	0.01	0	0.006	0.012	0.004
Cyclohexane	0.04	0	0.004	0.017	0.006
Benzene	0.32	0	0.327	0.473	0.003
Diethyl ether	0.38	2	0.081	0.610	0.013
Chloroform	0.40	0	2.44	3.0	0.004
Ethyl acetate	0.58	2	0.758	3.54	0.028
Water	0.70	Large	1.00	1.00	1.00

[a] Ratio of 5-VOT in solvent and aqueous phase determined by extraction of 5-VOT (0.75 ppm in 5 mL aqueous phase) twice with 5 mL solvent (11).
[b] Solvent strength parameter for adsorption chromatography on alumina (13).
[c] PASP = proton-acceptor solubility parameter (13).

Table 3. k' Capacity factors of 5-VOT determined by partition chromatography with weakly polar or nonpolar stationary phases and aqueous or nonaqueous eluants, 7.0 pH

Eluants	Stationary phases		
	Cyanopropyl[a]	Phenyl[b]	Octyl[c]
Hexane	—[d]	—[d]	—[d]
Hexane/CH_2Cl_2 (20 + 80)	5.9	4.6	2.6
CH_3Cl_3	2.9	2.0	1.3
CH_3CN/H_2O (25 + 75)	0.7	3.0	2.4
H_2O	1.9	12.7	12.5

[a] Zorbax CN, 5 μm, 150 × 4.6 mm (Dupont).
[b] Zorbax phenyl, 5 μm, 250 × 4.6 mm (Dupont).
[c] Lichrospher C8, 5 μm, 125 × 4 mm (Merck).
[d] Noneluted.

Table 4. k' Capacity factors of 5-VOT determined by adsorption chromatography on bare silica with aqueous or nonaqueous solvents, 7.0 pH

Eluants	Stationary phase
	Bare silica[a]
Hexane	—[b]
Hexane/CH_2Cl_2 (20 + 80)	16.3
$CHCl_3$	4.7
CH_3CN/H_2O (25 + 75)	1.3
H_2O	1.9

[a] Lichrosorb Si 100, 5 μm, 250 × 4 mm (Merck).
[b] Noneluted.

traction is carried out by shaking the tube with the aqueous and cyclohexanic phases for 2 min. A device is used to maintain regular and nonvigorous stirring (see *Apparatus*) to avoid the formation of a stable emulsion that could hinder recovery of the cyclohexanic phase. Stirring time necessary for complete extraction should be determined according to the apparatus. Under optimal conditions and working volume to volume, recovery is excellent (Table 5) when the concentration of 5-VOT in the aqueous phase is less than 50 ppm. Above this amount, extractions must be multiplied because the quantity of PMA dissolved in cyclohexane would be insufficient to achieve complexation of the entire 5-VOT.

PMA can take more concentrated solutions, obtained by increasing the polarity of the solvent or by changing PMA by a less polar complexing agent. These modifications must be well thought out because increasing the solvent's polarity diminishes selectivity of the extraction. Impurities with a polar nature may be extracted simultaneously with 5-VOT and not eliminated in the following steps (decomplexation, purification on solid phase). Moreover, the recovery from complexation and decomplexation could be modified. Work is currently underway in our laboratory to determine the influence of using solvents and complexing agents with different polarities in the extraction step. In reality, most of our sample analyses contain close to or less than 10 ppm 5-VOT. In this case, the results of Table 5 show that a reduced amount of the PMA solution may be used with respect to the extract. While retaining the volume of the PMA solution, the sample weight solution can be increased to concentrate 5-VOT during complexation, thus increasing the sensitivity (Table 1). Even if concentration by a factor of 10 is possible with standard solutions of 50 ppb of 5-VOT (Table 5), this is not the case with natural samples. Small measured additions of 5-VOT should verify the extraction efficiency.

Attempts to analyze the 5-VOT/PMA complex by LC on bare or CN, phenyl, and octyl silicas with nonaqueous eluants failed. We noted that this complex decomposes easily at the top of the chromatographic column. Consequently, we tried to develop a method to displace 5-VOT from the complex and analyze it according to the results in Table 3.

(b) *Decomplexation.*—Four compounds in an aqueous solution (iodide, thiocyanate, thiosulfate ions, and thiourea) were tested because of their strong capacity to form complexes with mercury and to release 5-VOT from PMA. The thiourea and thiocyanate ion only work at concentrated levels (0.5M and 10M), whereas the thiosulfate ion is efficient starting with 0.01M (Table 6). Recovery with the iodide ion (0.01M to 0.5M) was weak but consistent, which can be explained by the reactivity of the iodide ion with respect to the released 5-VOT. The obvious choice was to use thiosulfate because it requires little concentration for decomplexation (0.01M), thus reducing problems of elution in the following chromatographic step. Indeed, the thiourea, iodide, and thiocyanate ions give, at the beginning of the chromatogram, greater peaks whose "tail"

Table 5. Yield (%) of complex formation between 5-VOT and PMA according to volume of PMA solution used to extract 5-VOT from 1 mL aqueous solution

5-VOT concentration in aqueous solution, ppm	PMA solution, mL				
	0.050	0.100	0.200	0.500	1.000
50	—	20	32	75	99
1	70	73	94	100	100
0.05	70	100	100	100	100

Table 6. Recovery (%) of 5-VOT from cyclohexanic solution of complex PMA-5-VOT[a] by action of 4 decomplexing agents in aqueous solution[b,c]

Concentration, M	Decomplexing agents			
	Iodide	Thiocyanate	Thiourea	Thiosulfate
0.0001	10.2	3.4	9.1	10.9
0.001	38.0	3.5	17.3	66.9
0.01	42.5	10.7	26.1	93.3
0.1	41.8	21.8	37.1	97.1
0.2	45.2	30.8	45.8	102.5
0.5	44.3	36.2	85.0	96.4
1	—	46.7	—	93.5
10	—	77.0	—	—

[a] Obtained from 50 ppm aqueous solution of 5-VOT by action, volume-to-volume of saturated solution of PMA in cyclohexane.
[b] Ratio of released 5-VOT from PMA complex determined by liquid chromatography of 5-VOT in aqueous solutions of decomplexing agent (method described in *Experimental* and modified by using external calibration).
[c] Volume-to-volume with manual shaking.

disturbs detection and peak measures of 5-VOT. On the other hand, the thiosulfate produces little interference because of its much weaker absorbance at 241 nm and shorter retention time.

(c) *Complexation/decomplexation extraction properties.*—The complexation/decomplexation method of extraction offers the following advantages: 5-VOT can be transferred from its original aqueous medium to a very different polar organic environment (complexation), and then back again to the aqueous environment (decomplexation). Very few impurities are able to pass through this same route. Only the oxazolidine-2-thiones (OT) and compounds with the same reactivity with PMA or compounds soluble in both water and cyclohexane are extracted, making this operation highly selective.

5-VOT is quantitatively extracted because the affinity of PMA for 1,3-oxazolidine-2-thione is great. Thus, direct LC analysis of sow milk, using an external calibration, reveals a much lower 5-VOT content compared with an analysis of the same sample extracted with PMA (Table 7). This shows the presence, between 5-VOT and matrix, of bonds that are broken because of the great complexation constant between 5-VOT and PMA. Heating and acidification of the sample, generally carried out to improve 5-VOT extractions (5–10) by protein denaturation, are not necessary with PMA (Table 8). Finally, small measured additions of 5-VOT to the sow milk indicate that recovery of the extraction by PMA is entirely satisfactory (Table 9).

The purified extract containing only 5-VOT and thiosulfate ion can be analyzed directly by LC if the concentration of 5-VOT is superior to 0.5 ppm.

Nevertheless, because determination of the 5-VOT content is in the ppb range (3) for biological fluid or tissue analyses, we have studied means of eliminating the thiosulfate ion in the purified extract, while concentrating 5-VOT.

Purification on Solid Phase and Concentration

Different 5-VOT chromatographic tests with an aqueous eluant showed significant retentions on phenyl- and octyl-bonded silica (Table 3). Moreover, thiosulfate is a mineral ion whose retention times are much shorter on these apolar stationary phases, making them more suitable for the separation of 5-VOT from the thiosulfate ion. Using a system of cartridges packed with octadecyl-bonded silicas (see *Apparatus*) to prepare samples, 5-VOT and thiosulfate ion retentions were as expected. In other words, 5-VOT can be blocked in the stationary phase, eliminating the residual thiosulfate ion when washed with water, and eluting 5-VOT with a small amount of 70% acetonitrile in water (Figure 3). Thus, 5-VOT can be concentrated 4 or 5 times or more if the initial percolated volume is increased. When at least 2.5 mL extract is applied, 5-VOT elutes slowly and a part is lost when washed with water. In using an eluant containing more acetonitrile, precautions are required because the resulting 5-VOT eluate is, in turn, richer in acetonitrile than the composition of the mobile phase for LC analysis. At the injection time, this causes an imbalance in the chromatographic system (14), thus creating peak deformities and/or splittings, and a decrease in sensitivity.

LC analysis of the eluates obtained with acetonitrile-water (70 + 30) reaches a minimum detectable amount of 50 ppb (S/N = 4) for a sample weight of 0.2 g and an injection volume of 20 μL. To obtain a less than ppb value (Table 1), the last 2 parameters must be increased and the eluate concentrated by evaporation. An eluant stronger than the 70% acetonitrile in water (pure acetonitrile) can then be used at the moment of elution in the SPE step. It is eliminated during evaporation. The residue is then taken up by a small volume (50 μL) of 10% acetonitrile in water and chromatographed.

The mixture of the solvents is evaporated by nitrogen stream, which gives better results than a reduced pressure evaporation at 40°C. In the case of highly sensitive analyses, using plastic ware (tubes, pipettes, etc.) must be avoided because they contaminate the solution to be analyzed sufficiently to hamper chromatographic peaks.

During the complexation, decomplexation, purification, and concentration steps, volume recoveries varied when 5-VOT was being transferred from one phase to another. For this reason, it is difficult to find a satisfactory external calibration carried out with 5-VOT. We used an internal standard that underwent the same operations as 5-VOT, thus permitting precise measuring.

Table 7. 5-VOT values obtained for sow milk sample using liquid chromatography with direct injection following different treatments

Treatment	5-VOT, ppm[a]
Filtration, 0.22 μm	0.10 ± 0.03[b]
Protein precipitation[c]	0.07 ± 0.03[b]
PMA extraction[d]	0.82 ± 0.03[b]

[a] Method described in *Experimental* and modified by using external calibration.
[b] Average ± standard deviation calculated with 2 determinations.
[c] HCl addition (pH: 4.5) and filtration at 0.22 μm.
[d] Method described in *Experimental* and modified by not using internal standard.

Table 8. Effect of preliminary treatments of sow milk samples on efficiency of 5-VOT extraction with PMA

Treatment	5-VOT, ppm[a]
None	1.15 ± 0.04[b]
Heating[c]	1.11 ± 0.03[b]
Protein precipitation[d]	1.15 ± 0.01[b]

[a] Method described in *Experimental*, modified by introducing internal standard after sample treatment.
[b] Average ± standard deviation calculated with 2 determinations.
[c] 90°C for 10 min.
[d] HCl addition (pH 4.5). The precipitated proteins are not discarded.

Internal Standard

Because the internal standard is preferably introduced at the beginning of the analysis to reduce potential errors due to phase transfers, it should behave in the same way as 5-VOT during complexation, decomplexation, purification on C18, and concentration by evaporation. However, it must be separated in the chromatographic analysis. For this reason, an OT, 4,4-dimethyl-1,3-oxazolidine-2-thione (4,4-DMOT) (Figure 1), was specially synthesized (12). This compound has the same heterocyclic skeleton as 5-VOT, making its behavior identical during the various steps as indicated in Table 10. The slight polar differences between the vinyl and gem-dimethyl groups were used to an advantage for their LC separation.

Chromatographic Conditions

Because 5-VOT was found in the aqueous solution at the end of the sample preparation, a system with aqueous eluants and apolar stationary phases (phenyl- or octyl-bonded silicas) was found to be the best choice for chromatographic conditions (Table 3). A Lichrospher C8 column (5 μm 125 × 4 mm) was used for separation. Isocratic elution at room temperature with 10% (v/v) acetonitrile in water and a flow rate of 1 mL/min lets any thiosulfate ion separate in 10 min from 4,4-DMOT, 5-VOT, and 5-AOT (Figure 2). The latter compound, containing a supplementary methylene group compared with 5-VOT (Figure 1), comes from the degradation of the 2-hydroxypent-4-enyl glucosinolate. Far less of 2-hydroxypent-4-enyl glucosinolate is found in rapeseed than the precursor of 5-VOT (progoitrine). For this reason, 5-AOT is often overlooked in analyses of only 6 min.

The UV absorption spectra of the OT present a maximum molar extinction coefficient at 241 nm (ε = 16 000 L/mol cm^{-1}). However, LC detection can be done at 254 nm using a fixed wavelength detector with acceptable sensitivity (less than 50% reduction). The detection limit at 241 nm is around 0.2 ng of 5-VOT injected onto the column (S/N = 4) (Figure 4).

The identity of 5-VOT found in milk samples was confirmed by comparison with synthetic 5-VOT by liquid chromatography. Retention times and UV spectra determined by co-injection into a highly sensitive scanning UV detector did not reveal any minor differences.

Comments

Examination of this method shows that its use can be modified according to the needs of each laboratory. The advantages of this method are mainly in the extraction mode by complexation/decomplexation and the use of an internal standard. For high 5-VOT level samples (>500 ppb), the purification on C18 and concentration and evaporation steps can be eliminated, arriving at a very simple and rapid analysis without loss of precision. However, it is difficult to operate without the complexation/decomplexation steps because of their selectivity and extraction power. This method makes possible analyses in complex environments and, because of its nearly total elimination of impurities, it is practical and very effective.

Table 9. 5-VOT values obtained for 3 sow milk samples before and after 5-VOT addition

Milk sample	5-VOT added, ppm	5-VOT, ppm[a,b]		Rec., %
		Before	After	
A[c]	1.00	0.92 ± 0.04	1.91 ± 0.01	98
B[d]	1.00	2.01 ± 0.07	3.03 ± 0.08	102
C[c]	5.00	7.96 ± 0.10	12.86 ± 0.04	98

[a] Method described in *Experimental*.
[b] Average ± standard deviation.
[c] 2 determinations.
[d] 5 determinations.

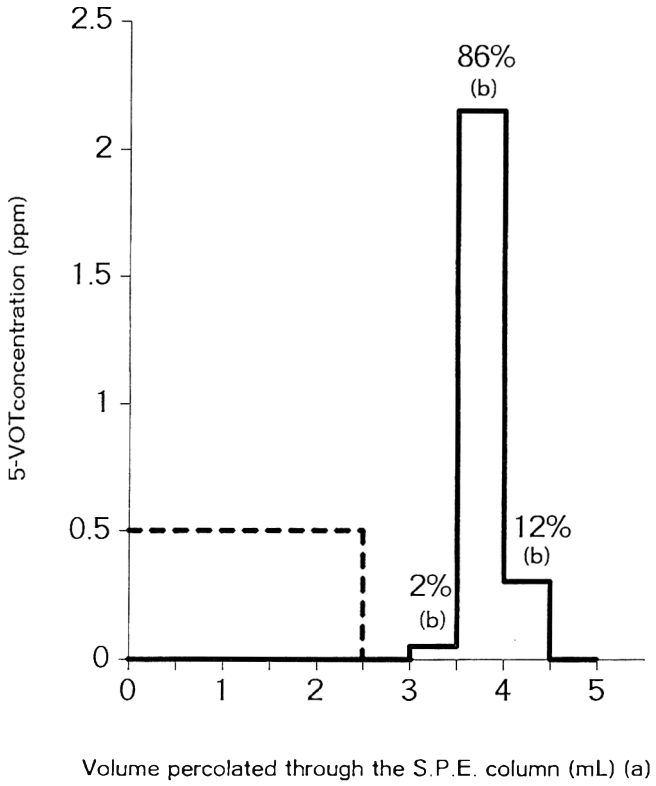

Figure 3. Purification and concentration of aqueous 5-VOT solution (0.5 ppm) on solid-phase extraction (SPE) column (200 mg of C18 bonded silica 40 μm). (— —) 5-VOT level in initial solution. (—) 5-VOT level in effluent. (a) From 0 to 2.5 mL; pour with 0.5 ppm 5-VOT solution. From 2.5 to 3.5 mL: wash with water. From 3.5 to 4.5 mL: elute with 70% acetonitrile in water. (b) Recovery of 5-VOT.

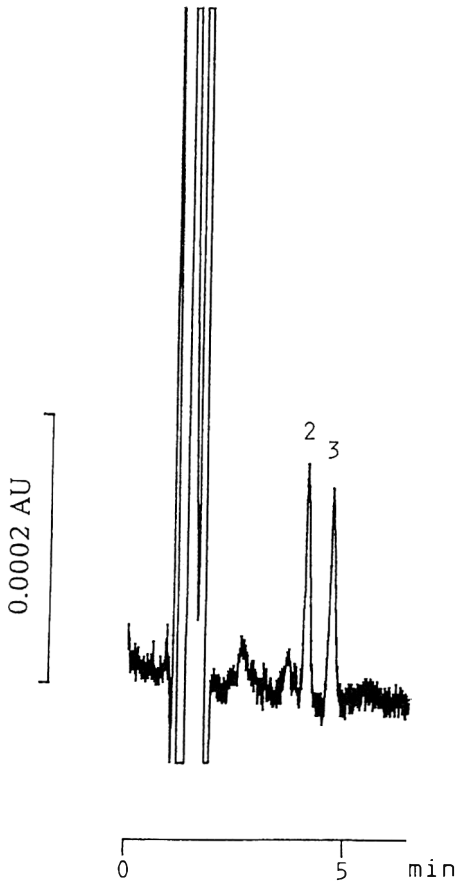

Figure 4. Chromatogram of aqueous standard solution of 4,4-DMOT and 5-VOT at 10 ppb. LC conditions: see *Experimental.* Peak identification: see Figure 2.

Conclusion

This simple method lets any laboratory analyze less than 1 ppb of a degradation product (5-VOT) coming from the most important glucosinolate found in rapeseed (progoitrin). We believe that this analytical procedure, which used complex samples of sow milk in the present study, may easily be applied to other biological fluids or tissues. This research is currently being carried out in our laboratory and will soon be published.

Table 10. Peak area ratios of 5-VOT and 4,4-DMOT (internal standard) given by LC of 1 ppm solution resulting from different preparation steps

Preparation step[a]	Peak area ratio
Direct injection in LC	1.18
Complexation-decomplexation	1.17
SPE concentration	1.20
Evaporation concentration	1.17

[a] Methods described in *Experimental.*

These results should contribute vital information to better understand the role of 5-VOT in the biological food chain. Nutritional sources containing glucosinolates could, thus, be developed for animal or human consumption.

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SUGARS AND SUGAR PRODUCTS

Evaluation of the Color of Some Spanish Unifloral Honey Types as a Characterization Parameter

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The color of 7 types of Spanish unifloral honey from rosemary, orange blossom, lavender, eucalyptus, sunflower, heather, and honeydew was investigated for its potential use as a characterization parameter. Colors were estimated by visual comparison with a Lovibond 1000 instrument, the readings of which were transformed into Pfund units. As an alternative method, the transmittances of liquid samples at selected wavelengths were measured, tristimulus values were calculated, and chromatic coordinates in the CIE-1931 (x,y,L) and CIE-1976 ($L^*a^*b^*$) color spaces were determined. The correlation coefficient between x and the Pfund grading was 0.958, but visual comparisons proved to be less objective and precise than CIE parameters; yet, analysis by visual comparison can be used by unskilled dealers and beekeepers because of its great simplicity. A stepwise discriminant analysis revealed that CIE-1976 ($L^*a^*b^*$) coordinates yield an overall proportion of accurately classified samples slightly better than that afforded by CIE-1931 coordinates (76 vs 71%). However, rosemary and lavender honeys were more accurately classed by using the CIE-1931 system. The results show that color determinations make a useful tool for helping to classify honeys.

Organoleptic properties have been used for a long time to distinguish honeys of different botanical origin. Some color ranges, aromas, and flavors are usually typical of a given floral type. The sensorial assessment of these properties is usually highly subjective.

The color of honey ranges over a continuum from very pale yellow through amber to dark reddish amber to nearly black (1) and can be assessed by a number of methods. The "universal melloscope" method, for example, which is rather empirical and arbitrary, was formerly used extensively by beekeepers in some European countries (2). The Pfund Color Grader, a visual comparison system developed by Sechrist (3), consists of a standard amber glass wedge with which the liquid honey contained in a wedge-shaped cell is compared visually. The color intensity of the honey is expressed as a distance along the amber wedge and usually ranges between 1 and 140 mm. This scale has been widely used by the honey trade. Brice et al. (4) developed a straightforward system of glass color standards, later adopted as an official method by the U.S. Department of Agriculture (USDA), that allows honey samples to be classed as water white, extra white, white, extra light amber, light amber, amber, and dark amber. One other scale based on I_2–KI solutions of different concentrations did not gain widespread acceptance because of the instability of the standards (5).

The Lovibond 1000 visual comparator is a straightforward instrument for measuring honey color in Pfund units in a shorter time than the Pfund Color Grader. In a collaborative study, White (6) recommended a color measuring method based on the use of a Lovibond 2000 visual comparator furnished with a disc carrying 6 circular color glasses that matched Brice's colors as far as possible. The method was adopted as official by AOAC (7).

The visual detection involved in all the above procedures does not allow small color differences to be sensed properly, particularly in samples not placed side by side.

The tristimulus methodology developed by the International Commission of Illumination (CIE) (8) was used by Brice to characterize the USDA color standards (4). Aubert and Gonnet (2) applied the CIE colorimetric coordinates obtained by the simplified method reported by Hardy (9) to 20 different unifloral honey samples and came to the conclusion that tristimulus measurements are more objective and precise than Lovibond-Pfund measurements for identifying some types of honey, although the latter should be kept as commercial references. On

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the other hand, honey is known to darken with storage time and increasing temperatures (1, 10–12), the rate of the process being influenced by the botanical origin (12).

Rodríguez (13) assessed the color of various Spanish honeys and reported 3 simplified equations for calculating CIE-1931 coordinates. Huidobro and Simal (14) compared the color of Galician and commercially available honeys.

The aim of this work was to evaluate the advantages and disadvantages of using color measurements to class Spanish unifloral honeys and comparing the results provided by the Lovibond-Pfund visual comparison system with the CIE methods (CIE-1931 and CIE-1976 $L^*a^*b^*$) (8, 15) based on measuring the transmittances of liquid honeys.

Experimental

Colors were assessed by the Lovibond-Pfund methodology and by the CIE-1931 or the more recent CIE-1976 ($L^*a^*b^*$), or CIELAB, methods, both based on the tristimulus values.

Apparatus

(a) *Lovibond 1000 color comparator.*—Obtained from Tintometer Ltd, furnished with 10 mm path length glass cells and 2 discs, each containing 9 color glasses providing a scale of 17 different values.

(b) *Spectrophotometer.*—Dual-beam, fitted to a chart recorder (Shimadzu UV-240, Shimadzu Co.).

Honey Samples

Honey samples reported to be unifloral were obtained from beekeepers, traders, and official services. They were produced between 1980 and 1987 in different Spanish regions; although some of them were crude, others had been subjected to some heating. Their botanical origin was first ascertained by melissopalynogical analysis according to the International Commission for Bee Botany (16), together with sensorial assessment of their flavor and aroma. Pollen analysis was inapplicable to honeydew honey; electrical conductivity (>800 μS/cm) had to be determined. The honey samples used were as follows: 21 from rosemary (*Rosmarinus officinalis* L.), 27 from orange blossom (*Citrus* sp. pl.), 17 from lavender (*Lavandula latifolia* Med.), 22 from sunflower (*Helianthus annuus* L.), 20 from eucalyptus (*Eucalyptus camaldulensis* Dehnh.), 17 from heather (*Ericaceae*), and 30 from honeydew honey (*Quercus* sp. pl.). The samples were homogenized by mild warming (ca 50°C) and shaking. A subsample of 20–30 g of each was removed, completely liquefied, and filtered through a 0.25 mm mesh sieve. Samples were analyzed for pollen as soon as received and for color 4–15 days after collected, if approved as unifloral.

Pollen Analysis

Slides were prepared without acetolysis (16) by centrifuging 10 g honey dissolved in 20 mL dilute sulfuric acid (5 g H_2SO_4/L) for 10 min at 2500 rpm. The supernatant liquid was decanted, and the sediment was washed twice with 10 mL distilled water and centrifuged. The sediment was put on a slide, sprouted over an area ca 2 × 2 cm, dried at 40°C, and mounted with stained glycerine gelatin. Pollen grains were identified and counted under the microscope. After 300 pollen grains were counted, they were classified in the following frequency classes: predominant pollen (more than 45% of the pollen grain counted); secondary pollen (16–45%); important minor pollen (3–15%); and minor pollen (<3%).

To ascertain the botanical origin, guidelines for interpretation of results from the *Methods of Melissopalynology* (16) were followed.

Table 1. Selected wavelengths (nm) for computing tristimulus values with illuminant C

X	Y	Z
435.5	489.4	422.2
461.2	515.1	432.0
544.3	529.8	438.6
564.1	541.4	444.4
577.3	551.7	450.1
588.7	561.9	455.9
599.6	572.5	462.0
611.0	584.8	468.7
624.2	600.8	477.7
646.0	627.2	495.1
Multiplying factors		
f_X	f_Y	f_Z
0.09806	0.10000	0.11814

Color Measurement

(a) *Visual comparisons.*—Each clear, liquid sample was poured into the cell of the Lovibond 1000 comparator to avoid entrapping air bubbles. Another cell filled with distilled water was used as reference. A honey disc was held in the comparator, and a circular colored glass was kept in the front of the reference as the disk was spun. Sample and glass (with the reference) were irradiated by an internal daylight lamp. The disc was spun until sample color matched the color of one of the glasses or was intermediate between the two. The corresponding Lovibond grading reading was then transformed to Pfund units (mm) by means of a scale supplied by the instrument manufacturer.

(b) *Spectrophotometric measurements.*—Clear honey samples were carefully poured into 10 mm path length glass spectrophotometer cells, and their transmittance spectra were recorded between 700 and 350 nm with water as blank. The transmittance of each sample was measured at the 30 wavelengths selected by Hardy (9), namely, 10 per tristimulus value (X,Y,Z) (see Table 1), and its tristimulus values were calculated from the following expressions:

$$X = \Sigma f_x T_x$$

$$Y = \Sigma f_y T_y$$

$$Z = \Sigma f_z T_z$$

Table 2. Distribution of color parameters in 7 Spanish unifloral honey types measured by visual comparison (Lovibond 1000 comparator, with results expressed in mm Pfund) and spectrophotometric methods (CIE-1931 and CIE-1976 $L^*a^*b^*$)

Parameter	Value	Honey type[a] Rosemary (21)	Orange (27)	Lavender (17)	Sunflower (22)	Eucalyptus (20)	Heather (17)	Honeydew honey (30)
mm Pfund	Mean	23	30	70	67.5	71	133	119
	Min	< 11	11	46–51	51–55	51	99	83
	Max	51	55	92–99	71–83	83	140	140
x	Mean	0.369	0.378	0.471	0.472	0.458	0.652	0.582
	Min	0.337	0.343	0.425	0.428	0.410	0.577	0.514
	Max	0.429	0.439	0.523	0.509	0.499	0.735	0.680
y	Mean	0.381	0.391	0.456	0.474	0.450	0.347	0.406
	Min	0.347	0.356	0.434	0.450	0.419	0.265	0.319
	Max	0.435	0.441	0.469	0.488	0.481	0.419	0.464
L (%)	Mean	61.4	67.7	43.3	48.43	31.85	2.73	9.59
	Min	34.12	44.94	23.62	29.58	17.66	0.002	0.86
	Max	84.14	85.86	65.41	62.84	44.37	9.89	24.28
$\lambda_{P\ (nm)}$	Mean	576.5	576	579	578	579	613	593
	Min	575	575	576	576	577	591	582
	Max	578	578	584	581	582	675	615
P (%)	Mean	33	39	79	86	76	99.9	98.6
	Min	15.6	19.4	64.3	67.7	62.5	99.3	93.3
	Max	63.3	68.1	90.8	99.0	89.7	100	100
a^*	Mean	-1.25	-2.26	6.03	1.88	3.79	16.7	28.46
	Min	-2.679	-4.396	-3.081	-4.424	-0.777	0.160	12.739
	Max	1.502	2.199	19.258	10.547	9.641	32.278	42.445
b^*	Mean	32.7	36.0	74.0	86.2	60.7	22.9	57.5
	Min	15.860	19.408	60.251	66.003	41.61	0.003	13.396
	Max	77.136	61.202	87.684	103.05	74.184	64.290	81.253
L^*	Mean	82.0	85.4	71.1	74.7	62.7	29.5	34.9
	Min	65.059	72.859	55.706	61.298	49.086	0.002	7.777
	Max	93.516	93.508	84.698	83.362	73.685	37.648	56.369

[a] The number of samples of each honey type is given in parentheses.

where T denotes transmittances and f is the multiplying factor listed in Table 1 for the standard light source C (6774 K).

(c) *CIE-1931 chromatic coordinates.*—The tristimulus values allowed the CIE-1931 chromatic coordinates x, y, and L to be readily calculated from the following expressions:

$$x = X/(X+Y+Z)$$

$$y = Y/(X+Y+Z)$$

$$L = Y$$

The projection of the point corresponding to the color of a given sample H_0 (x_0,y_0,L_0) on the (x,y) plane was located inside the chromaticity diagram. The predominant wavelength λ_P, which represents the psychological attribute designated as hue, was read on the boundary line by intersecting it with the straight line crossing the point (x_0,y_0) and the point corresponding to the standard illuminant (achromatic point). The purity (P) of that color is the ratio between the distances from the achromatic point to (x_0,y_0) and to the spectrum locus and is representative of its saturation.

(d) *CIE-1976 ($L^*a^*b^*$) chromatic coordinates.*—The CIELAB color space is more uniform than the CIE-1931 space and is becoming increasingly popular (17). Its rectangular coordinates (a^*, b^*, and L^*) are calculated from the following expressions:

$$a^* = 500[F(1) - F(2)]$$
$$b^* = 200[F(2) - F(3)]$$
$$L^* = 116[F(2) - 0.1379]$$

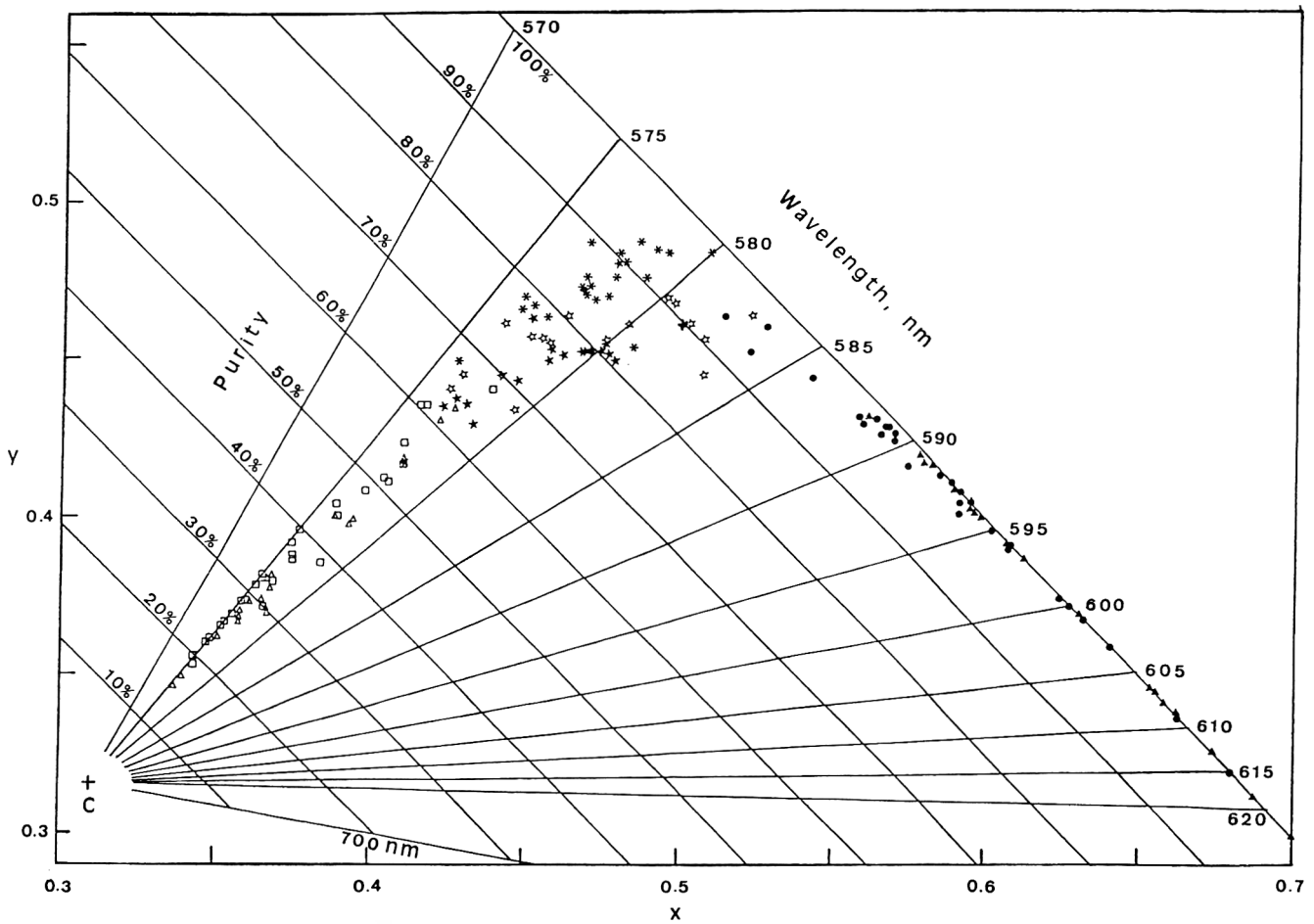

Figure 1. Localization area of liquid unifloral honeys on the chromaticity diagram (CIE-1931 color system). Δ: rosemary; □: orange blossom; ☆: lavender; ✱: sunflower; ✶: eucalyptus; ▲: heather; ●: honeydew honey; C: illuminant C.

where $F(i) = G(i)^{1/3}$ when G(i) > 0.008856; $F(i) = 7.787\,G(i) + 0.1379$ when G(i) < 0.008856; $i = 1,2,3$; and $G(1) = X/X_0$; $G(2) = Y/Y_0$; and $G(3) = Z/Z_0$.

X, Y, and Z are the tristimulus values of the sample, and X_0, Y_0, and Z_0 are those of the standard illuminant (98.04, 100.00, and 118.12, respectively, for illuminant C).

Results and Discussion

Pollen Analysis

The pollen spectra of the honey samples studied for color are briefly described in the following section, and the percentages are relative to pollen of nectar-producing plants.

Rosemary honeys contained 15–77% pollen of *Rosmarinus officinalis* L. Other taxons frequently identified were *Hypecoum* sp., *Rosaceae*, *Cistaceae*, *Cruciferae*, *Leguminosae* type *Ulex*, and *Thymus* sp. Orange honeys contained 10–46% pollen of *Citrus* sp. pl., although one sample reached 80%. Pollens of *Olea europaea*, *Cistus* sp. pl., *Cruciferae*, *Compositae*, *Leguminosae*, *Rosaceae*, and *Gramineae* were frequent. Lavender honeys contained 10–68% pollen of *L. latifolia* and pollens of *Helianthus annuus*, *Eucalyptus* sp., other *Compositae*, *Cistus* sp. pl., *Thymus* sp., *Leguminosae*, and *Hypecoum* sp. were usually found. Sunflower honeys contained 45–82% pollen of *Helianthus annuus*. Pollens of *Eucalyptus* sp., *Echium* sp., *Cistaceae*, *Leguminosae*, other *Compositae*, and *Cruciferae* were usually present. Eucalyptus honeys contained 62–98% pollen of *Eucalyptus* sp., which may be considered overrepresented. Taxons generally found as important minor or minor pollens in this honey type were *Echium* sp., *Cistus* sp. pl., *Compositae*, *Ericaceae*, and *Lavandula stoechas*. Heather honeys contained 49–80% pollen of *Ericaceae*. Other pollens usually found were those from *Cistaceae*, *Eucalyptus* sp., *Echium* sp., *Helianthus annuus*, *Leguminosae*, *Castanea sativa*, and *Rosmarinus officinalis*.

Color Measurement

Table 2 summarizes the results obtained from the color measurements made on the 7 types of unifloral honeys studied. By visual comparison, rosemary and orange blossom honeys were found to be the lightest (some samples yielded values below 11 mm Pfund, the lower limit of the scale), with extensive overlap. The colors of the eucalyptus, lavender, and sunflower honeys were generally darker (amber); they also overlapped, although some samples of the second type and most of the last could not be exactly matched to any Lovibond

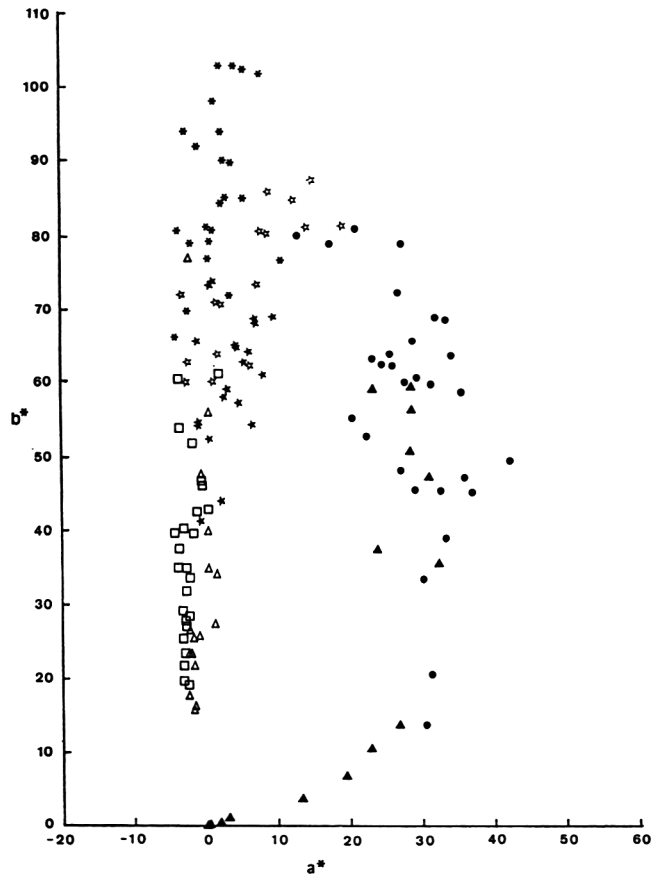

Figure 2. Localization area of liquid unifloral honeys on the (a^*,b^*) plane (CIE-1976 $L^*a^*b^*$ color system). Δ: rosemary; □: orange blossom; ☆: lavender; ✳: sunflower; ✳: eucalyptus; ▲: heather; ●: honeydew honey.

standard glass because they were brilliant yellow, which is not included in the system. This property distinguished most sunflower honeys from eucalyptus honeys. Heather and honeydew honeys were generally the darkest, with some samples yielding values of up to 140 mm Pfund (the upper limit of the scale). Their colors also markedly overlapped.

Table 2 also lists the mean and range of the rectangular coordinates of honey samples in the CIE-1931 color space, as well as the predominant wavelength (λ_P) and the purity or saturation (P). Figure 1 shows the projection of individual points on the (x,y) plane of the chromaticity diagram. It is a reversed V-shaped projection, enlarged at the apex.

Rosemary and orange blossom honeys yielded low values for x, relatively low values for y, and high values for the L chromaticity coordinate. Their λ_P values were in the 575–578 nm range, and P was relatively low (15–68%). Their colors overlapped markedly, although orange honey appeared to be slightly darker as a whole.

Lavender, eucalyptus, and sunflower honeys were quite similar. Their x and y coordinates were generally higher and their lightness (L) values were lower than those of rosemary and orange blossom honeys. Sunflower and some lavender honey generally yielded the highest y values and were bright yellows not matched by the Lovibond comparator. Their transmittance spectra usually showed 3 typical bands in the 400–500 nm region, consistent with the finding of other authors for sunflower honeys (2). Eucalyptus honeys, in the lower area of the enlarged apex (Figure 1), could be readily resolved from sunflower honeys by this procedure. However, both overlapped with lavender honeys.

The other 2 types of honey yielded x values greater than 0.51 (honeydew) and 0.57 (heather), and their y and L values decreased with increasing x. As can be seen from Figure 1, they had no characteristic predominant wavelength, and their purity was close to 100%.

The high scattering of the points yielded by each type of honey can be accounted for on the basis of the heterogeneity of the samples in relation to crop date, production region, age, and processing. However, this heterogeneity makes conclusions more independent of these variables.

Figure 2 shows the projection of the points yielded by honey samples onto the (a^*,b^*) plane. The corresponding data for groups are listed in Table 2. Rosemary and orange blossom honeys, with a^* and b^* values in the range −5 to 5 and 15–80, respectively, also overlapped extensively. Values of a^* are close to zero because X and Y have very similar values. Eucalyptus and sunflower honeys were clearly distinguished, with some exceptions, particularly through parameter b^* (40–75 for the former and 65–105 for the latter). Thus, they also overlapped with each other to some extent and with lavender honeys, although 30% of the samples of the lavender type yielded a^* values in the range 10–20, which was rather unusual for the other 2 types. The color points of honeydew honeys were spread on the right side of Figure 2, with a^* and b^* values in the ranges 10–41 and 10–81, respectively. Some heather honeys (the darkest ones) yielded a^* and b^* values lower than 30 and 10, respectively, so that they were clearly distinct from the other types of honey; however, the color of other samples overlapped with that of honeydew honeys. In any case, the color points in the (a^*, b^*) diagram are less extensively scattered (darker honeys lie closer to lighter honeys) than in the (x,y) projection.

Changes in L^* values were similar to those of L values in the CIE-1931 system, because L^* is a sole function of L (although L^* is always lower than L). Some color points corresponding to heather honey samples lie close to the center of the bottom of the CIELAB color space, i.e., the point 0,0,0.

Lovibond readings correlated well with x ($r = 0.958$) and L (and L^*) ($r = -0.920$), which is consistent with the findings of Aubert and Gonnet (2). Spectrophotometric methods are more objective and precise; therefore, it is possible to distinguish smaller color differences by spectrophotometric methods than by the Lovibond method. However, the latter can be used as reference for commercial purposes because of its simplicity and ease of application whenever high precision is not required. Obviously, improved color discs matching honey colors more closely on the basis of CIE colorimetric coordinates should be developed (2).

The honey color data provided by the chromatic coordinates in each CIE system were subjected to stepwise discriminant analysis (BMDP7M) (18), which revealed the best variables

Table 3. Classification matrixes of unifloral honeys by color measurement using functions obtained by stepwise discriminant analysis. (A) CIE-1931 color space; (B) CIE-1976 ($L^*a^*b^*$) color space

Group[a]	Percent correct	Number of samples classified into group[a]						
		ROS	ORA	LAV	SUN	EUC	HEA	HON
		A						
ROS	71.4	15	4	0	0	2	0	0
ORA	59.3	8	16	2	0	1	0	0
LAV	82.4	0	0	14	1	2	0	0
SUN	68.2	0	0	6	15	1	0	0
EUC	80.0	1	0	3	0	16	0	0
HEA	64.7	0	0	0	0	0	11	6
HON	70.0	0	0	2	0	0	7	21
Total	70.1							
		B						
ROS	57.1	12	6	2	1	0	0	0
ORA	74.1	5	20	1	0	1	0	0
LAV	70.6	0	1	12	2	2	0	0
SUN	90.9	0	0	1	20	1	0	0
EUC	80.0	0	0	3	1	16	0	0
HEA	64.7	0	0	0	0	0	11	6
HON	86.7	0	0	1	0	1	2	26
Total	76.0							

[a] Groups (types) of honeys are abridged as: ROS (rosemary), ORA (orange), LAV (lavender), SUN (sunflower), EUC (eucalyptus), HEA (heather), and HON (honeydew honey).

for distinguishing honey samples of different botanical origin to be x, y, and L, in that order, for the CIE-1931 system, and L^*, b^*, and a^* for the CIELAB system, also in that order. These variables were selected by the program in each step.

Table 3 lists, for both CIE color spaces, the proportions of honey samples correctly classified in their parent groups and the respective matrixes obtained from the functions yielded by the program.

The CIELAB coordinates yielded the higher overall number of successful classifications (76 vs 70.1%). Rosemary and lavender honey samples were classified more accurately through their x, y, and L coordinates, whereas eucalyptus and heather honeys were classified with roughly the same accuracy by both methods. The CIELAB variables seem to be the most valuable for classifying sunflower honeys (about 91% successful assignments) and the least valuable for rosemary honey (only 57.1% correct assignments). However, differences in correct classifications given by the 2 CIE methods are not statistically significant, as can be deduced from analysis of variance and *t*-test. The use of transformed variables of the chromaticity coordinates (e.g., reciprocals or squares) seems to improve the classification efficiency, although this approach requires more complex functions that do not always provide better results. Thus, the overall success rate was increased only from 70.1 to 74.0% by using L, z, y^2, 1/x, and 1/y instead of x, y, and L, but the efficiency in classing lavender honeys by the same procedure decreased from 82.4 to 76.5%.

The honeys assayed can be classified into 3 groups composed of (*a*) rosemary and orange blossom; (*b*) lavender, sunflower, and eucalyptus; and (*c*) heather and honeydew honeys. They were accurately classified in one of these groups in 89.6, 98.3, and 95.7% of all cases by using the (x, y, L) or (L^*,a^*,b^*) groups of variables. Some misclassifications can be ascribed to the mixing of naturally occurring honey arising from contemporary flowering in the same area (e.g., sunflower and lavender in Spain).

Although 100% correct classifying was not achieved in any case, CIE chromatic coordinates make a useful means of classifying honeys despite the variability of sample within each of the above groups. Other variables used for this purpose include pH, electrical conductivity, and chromatographic sugar spectrum, in addition to the traditional palynological analysis (19). Color assessment can, thus, be regarded as a useful complementary tool for determining the botanical origin of honeys.

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SUGARS AND SUGAR PRODUCTS

Internal Standard Stable Carbon Isotope Ratio Method for Determination of C-4 Plant Sugars in Honey: Collaborative Study, and Evaluation of Improved Protein Preparation Procedure

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A collaborative study of the internal standard isotope ratio method for measuring C-4 plant sugars in honey is reported. For 5 Youden pairs of samples containing 2–14% C-4 sugars, analyzed by 9 laboratories, mean s_r was 1.92% and s_R was 2.19%. Improved performance parameters for determination of $\delta^{13}C$ in honey are also presented. The method was adopted first action by AOAC International. Also presented is an improved purification procedure for the honey protein required for the internal standard method. The dialysis-precipitation procedure described is considerably less demanding of analyst's time and eliminates possible contamination of the preparation by indigenous yeasts and molds. No difference in $\delta^{13}C$ values was found between the collaboratively studied method and the method when used with the improved procedure to analyze 10 known pure honeys. The dialysis-precipitation procedure is recommended as a substitute for the repeated washing procedure described in the collaboratively studied method.

A new stable carbon isotope ratio procedure for testing honey for the presence of C-4 plant sugars (C-4S) uses the $\delta^{13}C$ value for the honey sample's protein as an internal standard for comparison with that of the original honey. A difference of −1.00‰ [the 4s level (1)] between $\delta^{13}C$ values of the protein and the honey is considered sufficient to indicate the presence of a significant amount of these sugars. Plants using the Hatch-Slack C4 carboxylic acid pathway in CO_2 fixation are referred to herein as C-4 plants. Examples are corn and cane.

A collaborative study of the method under AOAC guidelines (2) has been completed and is reported here. Eleven collaborators in 4 countries indicated willingness to join the study. Results were received from 9 collaborators.

Collaborative Study

Ten honey samples were selected from those analyzed in the earlier work; 9 are listed in Table 7 of that work (1). These samples represented unprocessed bulk honeys offered to the U.S. Department of Agriculture, Agriculture Stabilization and Conservation Service (USDA-ASCS) for the honey loan program. Because the new method is primarily intended for use when $\delta^{13}C$ values of a honey are less negative than −23.0‰, selection was restricted to samples analyzing from 0 to 15% C-4S, shown in that table as corn/cane content. Samples were liquefied to ensure homogeneity, and approximately 20 g portions of each were placed in polypropylene screw-cap bottles and labeled with 2-digit numbers selected from a table of random numbers. A practice sample from the same collection (approximately 35 g), with the $\delta^{13}C$ value found earlier, was included. Because protein isolation was the procedure under test, the collaborators were instructed to make a single protein isolation on each sample, to use their customary replication procedures (if any) for the isotope measurement, and to report both $\delta^{13}C$ for honey and protein and the calculated percent C-4S. Comments were solicited on the procedure, and descriptions of any deviations from the protein isolation protocol were requested.

Samples were assigned to Youden pairs according to the mean of values from all collaborators for percent C-4S after the results were returned. The data were analyzed as required (2).

Submitted for publication: collaborative study, February 6, 1991; evaluation of improved protein preparation procedure, October 30, 1991.

Recommendation (*1*) was approved by the General Referee and the Committee on Foods II and was adopted by the Official Methods Board of AOAC. See "Changes in Official Methods of Analysis" (1992) *J. AOAC Int.* **75**, January/February issue.

Table 1. Collaborative results for determination of $\delta^{13}C$ values for honeys and protein fractions (‰)

	Sample pair 1				Sample pair 2				Sample pair 3			
	46		84		70		98		63		64	
Coll.	Honey	Protein	Honey	Protein	Honey	Protein	Honey	Protein	Honey	Protein	Honey	Protein
1	-22.7	-22.4	-22.6	-23.1	-22.3	-23.5	-22.7	-23.7	-22.9	-23.9	-21.7	-22.8
2	-23.9	-23.3	-23.1	-23.6	-22.6	-23.9	-23.3	-23.9	-22.9	-23.6	-21.9	-22.9
3	-22.7	-23.1	-23.0	-22.9	-22.4	-23.1	-23.0	-23.5	-22.7	-23.7	-21.7	-22.6
4[a]	-23.3	-24.4	-23.6	-26.9	-23.0	-24.1	-23.6	-25.2	-23.3	-24.5	-22.3	-23.8
5	-23.0	-23.7	-23.4	-23.9	-23.0	-23.8	-23.4	-23.9	-23.1	-24.2	-22.1	-23.0
6	-22.5	-22.7	-22.7	-22.8	-22.1	-22.8	-22.7	-23.5	-22.5	-23.5	-21.4	-22.6
7	-23.3	-23.4	-23.6	-23.2	-23.0	-23.9	-23.5	-24.1	-23.3	-24.1	-22.3	-23.2
8	-22.9	-23.7	-23.3	-23.7	-22.6	-23.5	-23.2	-24.1	-22.9	-24.1	-22.1	-23.0
9[a]	-23.1	-23.6	-23.3	-23.1	-22.7	-23.1	-23.4	-23.8	-22.0	-23.7	-22.3	-22.9

	Sample pair 4				Sample pair 5			
	01		81		06		32	
Coll.	Honey	Protein	Honey	Protein	Honey	Protein	Honey	Protein
1	-21.8	-22.5	-21.6	-23.6	-21.7	-22.3	-22.4	-22.6
2	-22.2	-23.7	-22.2	-23.9	-22.1	-23.9	-22.7	-24.6
3	-22.1	-23.8	-22.0	-23.5	-21.9	-24.0	-22.4	-24.3
4[a]	-22.7	-24.3	-22.7	-24.3	-22.5	-25.0	-23.0	-25.6
5	-23.4	-24.0	-22.5	-23.9	-22.4	-24.4	-23.0	-24.9
6	-21.9	-23.8	-21.9	-23.2	-21.6	-24.0	-22.2	-24.3
7	-22.7	-24.0	-22.4	-23.8	-22.5	-24.3	-22.8	-24.5
8	-22.3	-23.8	-22.4	-23.9	-22.3	-24.4	-22.6	-24.6
9[a]	-21.1	-22.6	-22.4	-24.1	-22.4	-24.3	-23.4	-24.8

[a] Reported to 2 decimal places; rounded to 1.

991.41 C-4 Plant Sugars in Honey—Internal Standard Stable Carbon Isotope Ratio Method

First Action 1991

Method can be used to resolve uncertainty in interpreting $\delta^{13}C$ values between -23.4 and -21.5‰ (for citrus honey, -21.9 and -20.0‰). However, method is applicable to honey with any $\delta^{13}C$ value. (See Reference (1) for supporting data.)

Method Performance:

Range = 2.14–13.6%

s_r = 1.25–2.69; s_R = 1.97–2.69; RSD_r = 9.22–90.9%; RSD_R = 14.5–92.6%

A. Principle

Stable carbon isotope ratio value for protein isolated from a honey provides standard to which stable carbon isotope ratio value of whole honey is compared.

B. Apparatus

(a) *Centrifuge.*—With horizontal 4-head rotor for 50 mL tubes, to provide 1500 × *g*.

(b) *Isotope ratio mass spectrometer.*—VG 602E, or equivalent.

C. Reagents

(a) *Tungstic acid, sodium salt.*—10% aqueous solution.

(b) *Sulfuric acid.*—0.67N. Dilute 1.88 mL H_2SO_4 to 100 mL.

D. Determination

(a) *Honey.*—Determine $\delta^{13}C$ of honey test portion as in **978.17**.

(b) *Protein.*—If appreciable amounts of solid matter are present, strain honey through 100–150 mesh (nylon stocking material is excellent); any insoluble material heavier than water will contaminate protein precipitate.

Add 4 mL H_2O to 10–12 g honey in clear 50 mL centrifuge tube; mix well. Add 2.0 mL 10% sodium tungstate solution and 2.0 mL 0.67N H_2SO_4 to small test tube, mix, and immediately add to honey solution; mix well. Swirl tube in ca 80°C water bath until visible floc forms, with clear supernate. If no visible floc forms, or if supernate remains cloudy, add 0.67N H_2SO_4 in 2 mL increments, repeating heating between additions.

Fill tube with water, mix contents and centrifuge 5 min at 1500 × *g*, and decant supernate. Repeat washing, mixing, and centrifuging steps 5 times with ca 50 mL portions of water, thoroughly dispersing pellet each time.

Table 2. Collaborative results for determination of differences between $\delta^{13}C$ values for honey and protein and calculated content of C-4 plant sugars (C-4S)

	Sample pair 1				Sample pair 2				Sample pair 3			
	46		84		70		98		63		64	
Coll.	Diff.	%C-4S	Diff.	%C-4S	Diff.	%C-4S	Diff.	%C-4S	Diff.	%C-4S	Diff.	%C-4S
1	0[a]	0	-0.5	3.7	-1.2	8.7	-1.0	7.1	-1.1	7.0	-1.1	8.4
2	0	0	-0.5	3.6	-1.3	9.2	-0.6	4.2	-0.7	5.0	-1.0	7.6
3	-0.4	3.0	0	0	-0.7	5.2	-0.5	3.6	-1.0	7.1	-0.9	7.0
4	-1.1	7.5[b]	-3.3	19.2[b]	-1.2	7.6	-1.6	10.3	-1.2	8.1	-1.5	10.6
5	-0.7	5.0	-0.5	3.5	-0.8	5.7	-0.5	3.5	-1.1	7.6	-0.9	6.8
6	-0.2	1.5	-0.1	0.8	-0.7	5.3	-0.8	5.8	-1.0	7.2	-1.2	9.3
7	-0.1	0.7	0	0	-0.9	6.3	-0.6	4.2	-0.8	5.6	-0.9	6.7
8	-0.8	5.7	-0.4	2.9	-0.9	6.5	-0.9	6.3	-1.2	8.3	-0.9	6.8
9	-0.5	3.6	0	0	-0.4	3.0	-0.4	2.8	-1.7	12.1[b]	-0.6	4.5[b]

	Sample pair 4				Sample pair 5			
	01		81		06		32	
Coll.	Diff.	%C-4S	Diff.	%C-4S	Diff.	%C-4S	Diff.	%C-4S
1	-0.7	5.5	-2.0	14.4	-0.6	4.8[b]	-0.2	1.6[b]
2	-1.5	10.7	-1.7	12.0	-1.8	12.7	-1.9	12.8
3	-1.7	12.1	-1.5	10.9	-2.1	14.7	-1.9	13.0
4	-1.6	11.0	-1.6	11.0	-2.5	16.3	-2.6	16.4
5	-0.6	4.2	-1.4	9.9	-2.0	13.6	-1.9	12.5
6	-1.9	13.5	-1.3	9.6	-2.4	16.8	-2.1	14.4
7	-1.3	9.1	-1.4	9.9	-1.8	12.3	-1.7	11.5
8	-1.5	10.6	-1.5	10.6	-2.1	14.3	-2.0	13.4
9	-1.5	11.6	-1.7	11.8	-1.9	13.0	-1.4	9.3

[a] See text.
[b] Outlier by Grubbs test (2).

Place appropriate amount of protein in ceramic combustion boat similar to that used for honey samples. Combust protein by same method used for honey. If necessary to hold for later isotope ratio analysis, either transfer (Pasteur pipet) washed pellet with minimum amount of water to small vial, cap, and place in boiling water for 2 min, or dry protein for at least 3 h in ca 75°C oven.

Calculate apparent C-4 sugar content as follows:

$$\% \text{ C-4 sugars} = \{[\delta^{13}C_P - \delta^{13}C_H]/[\delta^{13}C_P - (-9.7)]\} \times 100$$

where $\delta^{13}C_P$ and $\delta^{13}C_H$ are $\delta^{13}C$ values, ‰, for protein and honey, respectively; and -9.7 is the average $\delta^{13}C$ value for corn syrup, ‰.

Report negative values from this calculation as 0%.

Sample is considered to contain significant C-4 sugars (primarily corn or cane) only at or above a value of 7%.

Ref.: (*1*) JAOAC **72**, 907(1989). (*2*) JAOAC **75**, May/June issue (1992)

Results and Discussion

Estimation of C-4 Sugar Content

Table 1 shows the $\delta^{13}C$ values for honey and protein; Table 2 gives the differences and the calculated percentage of C-4S. Positive values for the difference calculate to negative values for C-4S and are reported in Table 2 as zero. Performance parameters for percent C-4S are listed in Table 3. For all samples, mean s_r (repeatability, laboratory precision) is 1.92% C-4S; mean s_R (among-laboratories and within-laboratories precision) is 2.19% C-4S. Table 2 shows that a given difference does not correspond exactly to the same percent C-4S because the difference calculated between the protein standard and -9.7‰ depends on the value for the protein. The amount of C-4S in the samples covers only the range from approximately 2 to 14%, so the relative standard deviations are much higher for the samples of low content.

Determination of $\delta^{13}C$ in Honey

Performance parameters for the determination of stable carbon isotope ratio of honey, **978.17** (3), were included in the collaborative report on that method, which was based on results of 5 samples by 6 collaborators (4). A more extensive examination of this procedure may be made using the data for the 10 honey samples and 9 collaborators presented here. The samples were placed in Youden pairs in order of the means of their $\delta^{13}C$ values as seen in Table 4. This pairing differs from that used for the analysis for C-4S. The results of this calculation

Table 3. Performance parameters for collaborative study on determination of corn or cane sugar (C-4S) content of honey[a]

Statistic	Sample pair				
	84–46	70–98	63–64	81–01	06–32
No. laboratories	8	9	9	9	8
Mean, % C-4S	2.13	5.85	7.54	10.47	13.56
s_r	1.93	1.62	2.12	2.69	1.25
RSD_r, %	90.89	27.62	28.18	25.75	9.22
s_R	1.97	2.18	2.12	2.69	1.97
RSD_R, %	92.56	37.30	28.18	25.75	14.50

[a] Excluding outlying values (Table 2).

are shown in Table 5. The means of the performance parameters of the 5 pairs for the determination of $\delta^{13}C$ are $s_r = 0.19$, $s_R = 0.39$, $RSD_r = 0.84\%$, and $RSD_R = 1.72\%$.

Method **978.17** specifies the combustion system of Craig (5), which uses a tubular furnace with recirculation of gases. More recently, Sofer (6) described the use of combustion in sealed quartz tubes. Two of the collaborators reported using a recirculation system; all others used a sealed-tube combustion.

Collaborators' Comments

Collaborator 1 used a sealed-tube combustion procedure.

Collaborator 2 stated that the diluted honeys required 9 mL dilute acid and 3 mL tungstic acid to attain a clear supernate. The collaborator used sealed-tube combustion.

Collaborator 3 reported that 4 mL acid was required to form a clear supernate. The centrifuge accepted only 22 mL tubes; each portion was washed 10 times with 20 mL portions of water. Floc was dispersed with a Pasteur pipet, and the mixture was vortex-mixed. The pellet was dried on a watch glass overnight at 75°C. A sealed Vycor tube with CuO and Ag was used.

Collaborator 4 used a Schliegel and Vogel (7) recirculation combustion system.

Collaborator 5 used sealed-tube combustion with CuO and Cu.

Collaborator 6 centrifuged each sample in two 22 mL tubes. The final wash was filtered onto a 15 mm glass-fiber filter, air-dried at 73°C. Sealed-tube combustion was used.

Collaborator 7 used the Craig (5) combustion procedure.

Collaborators 8 and 9 used sealed-tube combustion.

Dialysis-Precipitation Procedure—Evaluation

The collaboratively studied method described (adopted as **991.41**) for isolating and purifying the honey protein (the internal standard) requires precipitation with tungstic acid, centrifugation, and removal of all carbohydrate material from the protein by 5 cycles of washing and centrifugation. Routine use of this method on commercial samples has emphasized its labor-intensive nature. There was organoleptic evidence of preprocessing fermentation in many of these honeys. In these samples, the protein isolate contained yeasts and other material, possibly contaminating it significantly. Preliminary centrifugation of the diluted honey before tungstic acid precipitation was subsequently used to remove these materials from several samples of such honeys. The $\delta^{13}C$ values of these protein preparations were compared with those from the same samples using the repetitive wash procedure of method **991.41**.

During the initial development of the internal standard method, dialysis was one of the preparative procedures first examined, followed by evaporation for recovery [(1), Table 1], but this was not further pursued. Since the collaborative study reported here was performed, an effort was made to eliminate the repetitive washing of the protein precipitate. Several of the commercial samples to which method **991.41** was applied were dialyzed and centrifuged before the protein was precipitated by tungstic acid, recovered by centrifugation, and washed once. $\delta^{13}C$ values for those samples were compared with values obtained earlier by method **991.41**. To demonstrate that results from this dialysis-precipitation procedure are not different from those of method **991.41** on pure honeys, 10 samples of

Table 4. $\delta^{13}C$ values of honeys paired for statistical analysis (‰)

Coll.	Pair 1		Pair 2		Pair 3		Pair 4		Pair 5	
	64	06	81	01	70	32	63	46	98	84
1	-21.7	-21.7	-21.6	-21.8	-22.3	-22.4	-22.9	-22.7	-22.7	-22.6
2	-21.9	-22.1	-22.2	-22.2	-22.6	-22.7	-22.9	-23.9	-23.3	-23.1
3	-21.7	-21.9	-22.0	-22.1	-22.4	-22.4	-22.7	-22.7	-23.0	-23.0
4	-22.3	-22.5	-22.7	-22.7	-23.0	-23.0	-23.3	-23.3	-23.6	-23.6
5	-22.1	-22.4	-22.5	-23.4	-23.0	-23.0	-23.1	-23.0	-23.4	-23.4
6	-21.4	-21.6	-21.9	-21.9	-22.1	-22.2	-22.5	-22.5	-22.7	-22.7
7	-22.3	-22.5	-22.4	-22.7	-23.0	-22.8	-23.3	-23.3	-23.5	-23.6
8	-22.1	-22.3	-22.4	-22.3	-22.6	-22.6	-22.9	-22.9	-23.2	-23.3
9	-22.3	-22.4	-22.4	-21.1	-22.7[a]	-23.4[a]	-22.0	-23.1	-23.4	-23.3

[a] Outlier by modified Cochran (Phillips, J.G., personal communication) and/or Grubbs (2) test.

Table 5. Performance parameters for determination of $\delta^{13}C$ of honey[a]

Statistic	Sample pair				
	64–06	81–01	70–32	63–46	98–84
No. laboratories	9	9	8	9	9
Mean, ‰	-22.07	-22.24	-22.63	-22.94	-23.19
s_r	0.06	0.41	0.07	0.34	0.07
RSD_r, %	0.27	1.83	0.31	1.50	0.30
s_R	0.34	0.52	0.32	0.42	0.35
RSD_R, %	1.52	2.34	1.42	1.81	1.49

[a] Exluding outlying values (Table 4).

known purity initially used to qualify the method (1) were analyzed by the new dialysis method.

Honey Samples

(a) *Commercial samples.*—Honeys offered for sale, with $\delta^{13}C$ values between -20.3 and -23.1‰, essentially the range used in the collaborative study. All had organoleptic indication of fermentation.

(b) *Pure samples.*—Ten of the 50 used earlier (1) with $\delta^{13}C$ values between -23.1 and -25.2‰.

Methods

(a) *Protein, repetitive wash.*—As described in method **991.41**.

(b) *Protein, dialysis procedure.*—Use cellulose dialysis tubing retaining proteins with MW >12 000, 30 cm × 25 mm (flat) (Sigma 250–9U is suitable). Hydrate tubing, closely tie 2 knots at one end. Heat 5–7 g honey to incipient boil (microwave oven is useful), add ca 3–5 mL H_2O, mix, place in sac, tie 2 knots in end, dialyze against running tap water for at least 16 h. Transfer contents of sac to 50 mL centrifuge tube, centrifuge 5 min at 1500 × *g*. Decant supernate into 100 mL beaker, discard pellet, add a fresh mixture of 6.0 mL 10% sodium tungstate and 6.0 mL 0.67N H_2SO_4. Heat on hot plate with stirring until visible floc forms, with clear supernate. Transfer to 50 mL centrifuge tube and centrifuge 5 min at 1500 × *g*. Discard supernate, disperse pellet thoroughly, fill tube with water, mix well, and centrifuge. Continue from "Place an appropriate amount..." in **991.41D(h)**.

Table 6. Internal standard isotope ratio analysis of commercial honey samples using several protein purification procedures

Sample	Honey $\delta^{13}C$, ‰	Protein $\delta^{13}C$, ‰		
		Original[a]	Yeast removed[b]	Dialysis[c]
1	-23.1	-24.0	—	-24.5
2	-22.2	-23.9	-25.0	-24.9
3	-23.0	-24.1	-24.7	-24.5
4	-23.0	-23.8	—	-24.8
5	-22.2	-24.0	-24.5	-24.5
6	-21.1	-25.1	—	-25.9
7	-21.2	-23.9	—	-24.2
8	-20.9	-24.7	-25.7	-25.5
9	-20.9	-23.6	-24.8	—
10	-21.8	-22.8	-23.9	—
11	-20.3	-23.8	—	-24.9
12	-22.9	-24.4	—	-25.3
13	-21.5	-23.8	-24.7	—
14	-21.9	-25.0	-25.3	—
15	-20.5	-23.3	-24.1	—
Mean		-24.01	-24.74	-24.90
SD		0.603	0.557	0.527

[a] Precipitation, repeated washing.
[b] With centrifugation before precipitation.
[c] Dialysis centrifugation procedure.

Evaluation Results and Discussion

Analytical results from 15 commercial samples are shown in Table 6. The simple removal of microscopic contaminants by centrifugation before precipitation, with no other change in

Table 7. Comparison of repeated washing and dialysis procedures for purifying honey protein for isotope ratio analysis

Sample[a]	Honey $\delta^{13}C$, ‰	Protein $\delta^{13}C$, ‰		
		Original[b]	Dialysis	Diff.[c]
28	-23.4	-23.7	-23.8	0.1
29	-25.1	-25.2	-25.0	-0.2
31	-23.1	-23.7	-23.8	0.1
32	-25.2	-24.4	-24.4	0.0
33	-24.0	-24.4	-24.2	-0.2
38	-24.8	-24.4	-24.5	0.1
42	-24.4	-24.8	-24.4	-0.4
43	-24.9	-25.2	-24.9	-0.3
45	-24.8	-24.7	-24.8	0.1
59	-25.2	-24.5	-24.8	0.3
Mean		-24.50	-24.46	-0.04
SD		0.519	0.430	—

[a] Sample numbers from Table 4, Reference 1.
[b] Values shown in Table 4, Reference 1.
[c] Value by original method minus that from dialysis procedure.

Table 8. Analysis of variance

Comparison	DF	F	p
Data in Table 6[a]			
Original vs yeast removal	17	8.26	0.011
Original vs dialysis	19	11.47	0.003
Yeast removal vs dialysis	7	0.13	0.736
Data in Table 7			
Original vs dialysis	19	0.04	0.853

[a] Using only paired values.

procedure (9 samples, "yeast removed" in Table 6), resulted in more negative values for $\delta^{13}C$ of the protein in every case. The mean difference for these 9 pairs is –0.83‰, roughly equivalent to nearly 6% additional C-4S. Ten samples were also analyzed by the dialysis procedure described above; results are shown in Table 6. For these 10 pairs, the difference between method **991.41** and the dialysis procedure results averaged –0.73‰. In 2 cases (1 and 4), the difference became large enough to change the status of the sample from "acceptable" to "contains C-4 plant sugar." For the 4 samples analyzed by the yeast removal-precipitation-5× wash procedure and also by the dialysis procedure described in (**b**) above, the average difference between results using these procedures for $\delta^{13}C$ of the protein is only 0.12‰.

Table 7 presents results for protein from 10 certified pure samples analyzed by the dialysis-precipitation procedure in (**b**) and by method **991.41**. The *t*-test indicates that the means for the 2 procedures do not differ significantly ($t = -0.57$, $P = 0.58$) when applied to known pure unfermented honeys. This is confirmed by the results from the analysis of variance (Table 8). Only paired values from Table 1 were included.

The dialysis-precipitation procedure for preparing honey protein for isotope ratio analysis has no significant effect on the $\delta^{13}C$ values of the protein when applied to pure honeys. Therefore, it may be substituted for the repeated washing procedure described in method **991.41**.

Recommendations

(*1*) We recommend that the internal standard stable carbon isotope ratio method for estimating the content of C-4 plant sugars in honey as described herein be adopted first action.

(*2*) We recommend that the performance parameters for determination of the stable carbon isotope ratio of honey (method **978.17**) obtained from this study be added to the current description of that method, that the title be changed to "C-4 Plant Sugars in Honey," that a suitable description of the Sofer (6) procedure be included as an alternative combustion method, and that the final paragraph under **978.17** be deleted and the following added: "Samples with $\delta^{13}C$ less negative than –23.5‰ are considered to contain significant amounts of C-4 plant sugars only after application of internal standard isotope ratio method (**991.41**) so indicates."

(*3*) We further recommend that the dialysis-preparation procedure for preparing honey protein for isotope ratio analysis be substituted for the repeated washing procedure used in the method described here in the first recommendation.

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VETERINARY ANALYTICAL TOXICOLOGY

Rapid Whole-Blood Cholinesterase Assay with Potential Use for Biological Monitoring During Chemical Weapons Disposal

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A protocol is described to determine whole-blood cholinesterase activity using an automated high-speed centrifugal analyzer. Throughput rate was increased 10-fold (from 6 to 60 samples/h), and precision, as measured by a reduction in intra-aliquot variation, was significantly improved (from 4.7 to 3.0%) by using an automated system rather than a manual system. Similar cholinesterase activity was determined for samples analyzed by both the automated and manual protocols, and interlaboratory comparison indicated no change in accuracy. The lower limit of detection was estimated to be 0.10 μmol/mL/min. The 10-fold increase in throughput facilitates use of the automated protocol when many analyses must be performed in a limited time.

Improvements in the ease and throughput of cholinesterase (ChE) assay methods are desirable and necessary in emergency situations [e.g., episodes of suspected accidental mass organophosphate (OP) pesticide poisoning in livestock and wild species, and biological monitoring in conjunction with the Chemical Stockpile Disposal Program (CSDP) of the U.S. Army]. The U.S. stockpile of aging unitary chemical warfare agent-munitions poses a calculated risk in both storage and disposal because some are powerful ChE inhibitors and are several orders of magnitude more potent than OP insecticides (1, 2). CSDP has been mandated by the U.S. Congress and is described by Carnes (3) and Carnes and Watson (4). The U.S. unitary stockpile is distributed primarily among 8 continental U.S. sites (2), most of which are adjacent to agricultural areas and 2 of which have on-site grazing lease programs for livestock.

Munro et al. (5) propose the use of blood ChE monitoring in sentinel species during disposal for such purposes as (*a*) indicating sublethal agent exposure in the event of a nerve agent release where timely deployment of low-level chemical monitors is not feasible, (*b*) providing decision criteria for treatment and disposition of livestock and pets during reentry and/or restoration after off-site contamination, (*c*) providing supplemental indication of safe conditions for human and animal reentry, and (*d*) biologically monitoring routine facility operations. An unplanned release of nerve agent would necessitate a rapid screening assay capable of analyzing samples from many animals.

Our laboratory staff programmed an automated system that uses a high-speed centrifugal analyzer with robotic pipettor to assay whole-blood ChE activity [combined influence of acetylcholine acetylhydrolase (EC 3.1.1.7) and acylcholine acylhydrolase (EC 3.1.1.8)] by the method of Ellman et al. (6), following procedures similar to those described by Harlin and Ross (7). The method described by Harlin and Ross (7) was adopted as the official first action enzymatic–spectroscopic method for determination of ChE in whole blood by AOAC (8). This paper presents our ChE protocol for the automated system in comparison with a manual spectrophotometric system, along with an assessment of assay precision and reproducibility.

Experimental

Samples

Staff of the University of Tennessee College of Veterinary Medicine (Knoxville, TN) collected whole-blood samples (N = 266) from Holstein dairy cattle located at the Southeastern Regional Correctional Farm in Pikeville, TN. Staff of the Animal Sciences Department, Virginia Polytechnic Institute and State University (Blacksburg, VA), collected whole-blood samples (N = 401) from sheep [whiteface cross (1/2 Dorset, 1/4 Finn, 1/4 Rambouillet)] located at the Animal Sciences Sheep Farm, Blacksburg, VA. All blood samples were collected from normal, healthy animals that were specially managed to avoid exposure to OP compounds. To eliminate clotting, blood samples were collected in 10 mL vacutainer tubes containing EDTA or in EDTA-washed syringes and then transferred immediately to 10 mL vacutainer tubes containing EDTA. Samples were refrigerated in Styrofoam containers with ice packs and/or a refrigerator and delivered to our laboratory within 24 h after collection. Whole-blood ChE activity was determined on the arrival date by a protocol previously established (7) for a manual system and a protocol

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developed in our laboratory for an automated system. The Harlin and Ross (7) protocol was modified slightly for our procedures (see below).

Apparatus

The COBAS FARA (Comprehensive Bioanalytical System, Flexible Automation for Random Analysis, Roche Diagnostic Systems, Nutley, NJ) system is a single unit high-speed centrifugal analyzer. It uses a microprocessor to control robotic pipettor arms capable of randomly selecting and loading several reagents, samples, diluents, and pretreatment solutions into a 30-place disposable plastic cuvet rotor. Each cuvet has 2 compartments, and the ingredients are mixed as the rotor accelerates. Absorbance is measured by placing cuvets horizontally in relation to the light path (9). The manual system used for the study was a Response series UV-VIS spectrophotometer (Gilford Systems, Ciba Corning Diagnostics Corp., Oberlin, OH).

Response Spectrophotometer Protocol

Whole-blood samples were analyzed manually by a modification of the spectrophotometric method described by Harlin and Ross (7). Briefly, the vacutainers containing blood samples were placed on a mechanical mixing table or were gently inverted by hand for ca 3–5 min to thoroughly mix the blood. A 1:1000 dilution of blood was prepared as follows: 0.1 mL of mixed blood was added to a 30 mL glass test tube containing 10 mL concentrated phosphate buffer (pH 8.0), and the blood–buffer solution was swirled to ensure thorough mixing. A 0.3 mL aliquot of blood–buffer solution was transferred to each of 4 polystyrene cuvets (1 × 1 cm, Kartell Corp., Milan, Italy) containing 2.7 mL concentrated phosphate buffer (pH 8.0). These sample–buffer dilutions are a modification of the protocol described by Harlin and Ross (7). Their protocol calls for diluting 0.01 mL sample in 10 mL phosphate buffer or 0.1 mL sample in 100 mL phosphate buffer; then, 3 mL diluted sample is transferred to a cuvet. Our modification results in the same sample dilution (1:1000) but reduces potential measuring error (measuring 0.1 vs 0.01 mL) and reduces the amount of phosphate buffer needed.

After adding 0.05 mL 0.1M dithiobisnitrobenzoic acid (DTNB) to each cuvet, 0.02 mL 0.075M acetylthiocholine iodide (ATCI) was added to 3 of the 4 cuvets. The fourth cuvet, without ATCI, served as a blank. The 4 cuvets were placed in a Response spectrophotometer cuvet holder with the blank in Position 1. The cuvets were covered with parafilm and inverted several times to mix the contents, the parafilm was removed, and the cuvet holder with cuvets was placed in the spectrophotometer. After a 1 min delay, spectrophotometric readings at 412.0 nm were recorded continuously for 6 min at a constant temperature of 25°C. The increase in absorbance at 412 nm on addition of ATCI and DTNB is attributable to the formation of yellow product (5-thio-2-nitrobenzoic acid). ChE activity is expressed as micromoles of product formed/mL/min (see Harlin and Ross (7) for calculations and reagent specifications).

COBAS Protocol

Blood samples in vacutainer tubes were mixed as described above. A 50 μL aliquot of blood was transferred to a 700 μL COBAS sample cup containing 450 μL concentrated phosphate buffer (pH 8.0), mixed, and placed in the appropriate sample rack. Next, 3 μL diluted sample, 260 μL DTNB reagent, 30 μL distilled water filtered through a 0.22 μm Millipore filter (Millipore Corp., Bedford, MA), and 10 μL ATCI were mixed. The final concentration of all reagents was the same as for the manual method. Spectrophotometric readings (first reading at 10 s followed by 9 additional readings at 10 s intervals) were made at 412 nm and a constant temperature of 25°C. For our purposes, the assay was repeated 3 times for each sample dilution. Aliquots of a calibrator serum specific for the COBAS served as a control that was assayed with each sample rack. (COBAS calibrator serum is an in vitro diagnostic control for clinical chemistry assays; it is prepared from human serum, to which a chemical and human and animal tissue extracts are added.) COBAS operating parameters are available from the authors upon request.

Precision and Reproducibility

We were interested in determining the precision and reproducibility (as an indication of accuracy) of our assay in addition to the efficiency and speed at which samples can be analyzed. We compared the precision (repeatability) of the assay using the COBAS protocol to that of the assay using the Response spectrophotometer protocol. This was done in 2 ways: by calculating the percent difference between the greatest and least ChE activity in triplicate aliquots of whole-blood samples collected from sheep and dairy cattle and by the repeated assay of a single sample using both instruments. Differences between instruments were determined by PROC TTEST procedures (SAS Institute Inc., Cary, NC).

Because no samples with known ChE activity were available, we determined reproducibility (as a measure of accuracy) by 2 methods. The ChE activities of 84 blood samples (40 sheep and 44 dairy cattle) determined by the COBAS protocol were subtracted from the activity values obtained from these same samples by the Response spectrophotometer protocol. We then determined the probability that the resulting difference was statistically non-zero (Student's paired *t*-test, SAS Institute Inc.). The Pearson's correlation coefficient between results by COBAS and by the Response spectrophotometer was determined. Next, we compared the results of repeated analysis of a single sample (calibrator serum) on both instruments. In addition to the above evaluations, assay results of 6 samples analyzed in our laboratory were compared with those obtained by the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Illinois.

Results and Discussion

The throughput rate for determining whole-blood ChE activity by the automated COBAS protocol was increased 10-fold (from 6/h to 60/h) over the manual Response spectro-

Table 1. Mean percent difference between maximum and minimum whole-blood ChE activity in sheep and cattle determined by an automated (COBAS) vs manual (Response) spectrophotometric system [modified Ellman assay; triplicate aliquots of whole blood drawn from whiteface crossed sheep (1/2 Dorset, 1/4 Finn, 1/4 Rambouillet) and Holstein cattle]

Species	COBAS ($\bar{X}$ ± SE)	Response ($\bar{X}$ ± SE)
Sheep	3.3 ± 0.12	5.1 ± 0.27
	(N = 282)	(N = 119)
Cattle	2.5 ± 0.14	4.3 ± 0.24
	(N = 146)	(N = 120)
Overall within-triplicate difference	3.0 ± 0.10 (N = 428)	4.7 ± 0.18[a] (N = 239)

[a] P = 0.0001, PROC TTEST.

photometer protocol. In addition, precision was significantly increased and accuracy (as measured by reproducibility) was not changed.

The percent within-triplicate difference in whole-blood ChE activity observed in randomly selected sheep and cattle blood samples was significantly reduced (P < 0.001), from 4.7 to 3.0%, when the COBAS assay was compared to the Response spectrophotometric assay (Table 1). The results for test of equality of variance (PROC TTEST) also indicated that the within-triplicate variances in whole-blood ChE activity determined by the COBAS and Response spectrophotometer assays were significantly different (P < 0.001). The ChE activity means determined from repeated analyses of a single sample (calibrator serum) by both the COBAS protocol and Response spectrophotometer protocol were similar, although a greater variance was observed in results obtained from the Response spectrophotometer protocol (Table 2, Figure 1). The mean within-triplicate difference observed in samples analyzed by the Response spectrophotometer protocol (4.7%) was similar to the mean within-duplicate difference (5.6%) reported by Harlin and Ross (7) in aliquots of a sample analyzed by different laboratories (5).

The increased precision obtained with the COBAS protocol (1.7%) is desirable and indicates greater sensitivity; however, it has little effect on the interpretation of exposure. Our research indicates that individual whole-blood ChE activity from healthy animals with no OP exposure may vary by approximately 30% through time. In addition, when clinical signs of acute poisoning are evident, usually whole-blood ChE activity is depressed by 80% of normal (10); approximately 50% depression of whole-blood ChE activity is considered diagnostically significant (in the absence of individual baseline data) (11).

Our results indicate that good reproducibility in whole-blood ChE assays is obtained by the Ellman procedure and COBAS instrumentation. There was no statistical difference in mean ChE activity determination in a single sample analyzed repeatedly by 2 different instrument protocols (COBAS and Response spectrophotometer) (Table 2, Figure 1). In addition, whole-blood ChE activities determined from 84 samples by the COBAS protocol and from those same samples by the Response spectrophotometer protocol were significantly correlated (Pearson's correlation coefficient = 0.96, P < 0.001). However, the mean difference (−0.07) between COBAS and Response spectrophotometer results for whole-blood ChE activity determined from these 84 samples was significantly different from zero (P < 0.001; Table 3). This statistical analysis suggests that the mean ChE activity obtained by the COBAS protocol (1.87 μmol/mL/min) tends to be slightly greater than that obtained by the Response spectrophotometer protocol (1.80 μmol/mL/min). However, this difference was less than 4%, and, as indicated previously, normal variability in whole-blood ChE activity would require a decrease in blood ChE activity of approximately 50% before OP poisoning would be considered. Therefore, the difference observed between instruments is of academic (and/or statistical) rather than practical importance (i.e., results obtained from both instruments are biologically similar and would result in similar diagnostic interpretations). In addition, there was no significant difference (P = 0.274) in assay results between samples analyzed in our laboratory and at the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Illinois, using manual instrumentation. Results of clinical studies using the COBAS protocol indicated a lower limit of detection (approximately 0.10 μmol/mL/min) that was similar to the detection limit reported by Harlin and Ross (7).

Our assessment is that the COBAS protocol provides a reliable means for determining whole-blood ChE activity in domestic species (whole blood from cattle, horses, and sheep have been assayed) and presumably wild species and humans.

Table 2. Mean ChE activity (μmol/mL/min) of a single sample[a] analyzed repeatedly using an automated system (COBAS) and a manual system (Response spectrophotometer)

Method	Replications	Mean	SE	Min.	Max.	Prob. > |T|[b]
COBAS	54	1.4	0.007	1.31	1.5	0.77
Response spectrophotometer	48	1.39	0.014	1.21	1.61	—

[a] Calibrator serum.
[b] Probability that the true means from analysis using the COBAS (1.4 μmol/mL/min) and Response spectrophotometer (1.39 μmol/mL/min) are equal (Student's paired t-test).

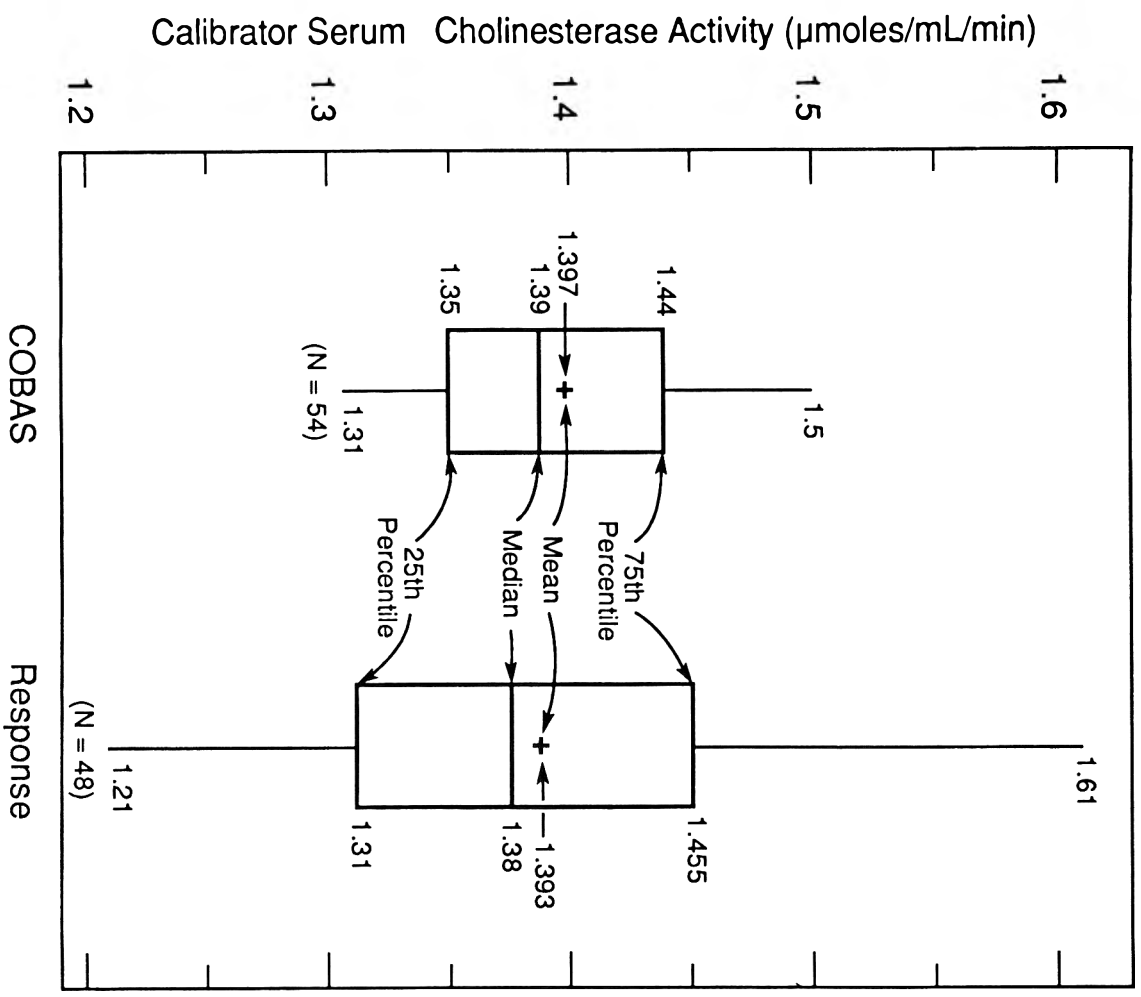

Figure 1. Statistical box plots of ChE activity from repeated assays of a single sample (Calibrator serum) by using protocols for COBAS and Response spectrophotometer instrumentation. Extreme values determined by COBAS (1.31 and 1.5) and Response spectrophotometer (1.21 and 1.61) protocols are indicated.

The COBAS protocol increased sample throughput rate 10-fold and significantly increased assay precision compared with the manual system tested without any apparent loss of accuracy. We consider the resulting improvements in speed and efficiency to be valuable for determining whole-blood ChE activity in emergency situations where large numbers of assays would be required over a relatively short period of time.

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Table 3. Mean whole-blood ChE activity (μmol/mL/min) determined in 84 samples (40 sheep[a], 44 Holstein dairy cattle) analyzed by the Ellman method using automated COBAS and manual Response spectrophotometer instrumentation

Spectrophotometric method	N	Mean	SE	Min.	Max.	Prob. |T|[b]
COBAS	84	1.87	0.077	0.96	3.61	
Response spectrophotometer	84	1.80	0.071	0.86	3.38	
Difference[c]	84	-0.07	0.021	-0.58	0.46	<0.001

[a] Whiteface crossed sheep (1/2 Dorset, 1/4 Finn, 1/4 Rambouillet).
[b] Student's paired *t*-test.
[c] Difference between Response and COBAS for each sample.

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VETERINARY ANALYTICAL TOXICOLOGY

Development and Validation of a Multiresidue Method for β-Agonists in Biological Samples and Animal Feed

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An analytical strategy for the detection of β-agonists containing either an *N-tert*-butyl or *N*-isopropyl group is described. Extract purification is based on immunoaffinity chromatography; final detection, identification, and determination are by gas chromatography/mass spectrometry. To prepare the immunoaffinity chromatography materials, polyvalent antibodies are raised against clenbuterol and cimaterol. The immunoglobulin G fractions of the corresponding rabbit antisera are isolated and coupled onto an activated Sepharose™ matrix. The prepared columns gave quantitative recovery for a large number of structurally related β-agonists at the 200 ng level. To quantitate and control false-negative results, isotope-labeled internal standards are used. For animal feed and bovine urine, analytical recoveries were close to 100% for all compounds except salbutamol (56 ± 5%). Recoveries from liver in the range of 60–70% (salbutamol, 40–50%). Limits of detection, based on the corresponding most abundant ion, were in the range of 0.05–0.2 μg/kg or μg/L. Limits for identification, based on the simultaneous detection of 4 diagnostic ions, were in the range of 1–2 μg/kg or μg/L. Repeatability and within-laboratory reproducibility, expressed as percent relative standard deviation, were all well below 15%, which fulfills the EC criteria for reference methods. The multiresidue method is an efficient, cost-effective way to determine illegal growth-promoting *N-tert*-butyl and *N*-isopropyl phenylethanolamines.

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Compounds with hormonal or thyreostatic action were prohibited for use within the European Community (EC) in 1989 (1). In practice, completely eradicating the illegal use of anabolic compounds has been difficult. A shift to a relatively new class of compounds (2), the phenylethanolamines, primarily developed as human or veterinary drugs working on the β_2-adrenergic receptor, has been observed on the black market since 1988 (3). The first of such compounds for which the large-scale misuse as a growth-promoter was observed in the EC was clenbuterol (Figure 1). Within a few months, The Netherlands and other EC countries developed and implemented an effective control strategy, and large-scale monitoring programs were started. However, in spite of the efficiency of the methods used and the intent of the EC legislation, the use of β-agonists was not eradicated; a variety of new, illegal related compounds appeared on the black market. One of the most important is salbutamol, a traditional out-of-patent human and veterinary drug.

The risk of these compounds for the consumer became apparent after reports from Spain that a serious outbreak of poisoning due to the consumption of bovine liver was related to residues of clenbuterol (4). For several β-agonists, methods were developed related to their therapeutic use in humans, e.g., salbutamol (5, 6), or their intended use as veterinary drugs, e.g., clenbuterol (7). After the use of β-agonists as growth promoters became apparent, other groups developed analytical methods for clenbuterol (8, 9) and for simultaneous determination of several compounds (10–12).

The objective of the present study was to develop and validate a flexible multiresidue method for a large group of β-agonists in a variety of matrixes. Additional goals were to process at least 10 unknown samples per day and to adhere to EC guidelines for reference methods for compounds with hormonal or thyreostatic action (13–15). Earlier studies in our laboratory showed that immunoaffinity chromatography (IAC) is suitable for extract purification (16, 17). When combined with benchtop gas chromatography/mass spectrometry (GC/MS), an analytical tool is obtained for routine control and monitoring as well as for confirmatory and reference analyses.

The majority of β-agonists known to be used for fattening veal calves and cattle contain either an *N-tert*-butyl or an *N*-isopropyl group, e.g., cimaterol (Figure 2). The method described here is based on IAC, using antibodies against a representative

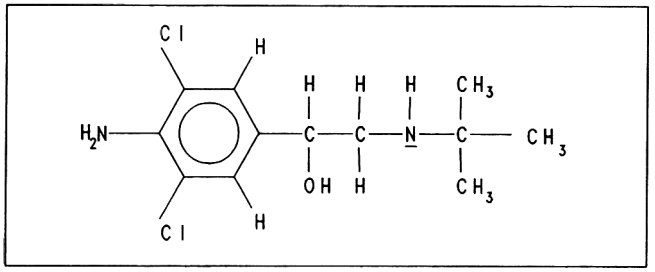

Figure 1. Molecular structure of clenbuterol.

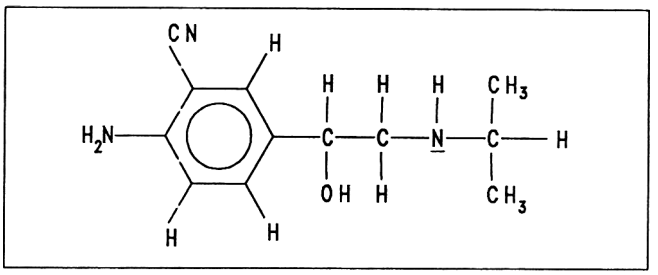

Figure 2. Molecular structure of cimaterol.

of each group. The combination of these antibodies allows the simultaneous isolation of a whole family of such compounds. Final detection and identification are based on GC/MS. For quantitation and quality control, isotopically labeled internal standards are used. The method is applicable for biological matrixes, such as urine and liver, and for animal feed, such as milk replacers and premixes. Development and validation of the analytical procedure is described.

Experimental

Materials

All chemicals, including standards and solutions, are of defined quality and subjected to a quality control program. Pure chemicals are of Pro Analyse quality or better. Water is double-distilled.

(a) *Reference compounds.*—See Table 1. Confirm identity of all standards by GC/MS and/or Fourier transform infrared (FTIR); check purity by liquid chromatography and/or thin-layer chromatography. If necessary, salts of these compounds or other related β-agonists may be used.

(b) *Stock solutions.*—1 mg/mL. Store in the dark at -20°C for up to a year.

(c) *Working solutions.*—Store in the dark at 4°C for up to 3 months.

(d) *Internal standards.*—For purposes of quality control and accurate quantitation, use labeled internal standards clenbuterol-d_6 and/or salbutamol-d_6 (donated by RIKILT, Wageningen, The Netherlands).

(e) *Bovine serum albumin (BSA).*—Sigma Chemical Co., St. Louis, MO 63178.

(f) *Immunogens.*—Prepare as described by Yamamoto and Iwata (18) by coupling diazonium–clenbuterol and diazonium–cimaterol, respectively, to BSA.

(g) *Antisera.*—Used to prepare IAC matrixes. Immunize New Zealand rabbits 4 times over 5-month period with 2 mg immunogen each time. Isolate immunoglobulin G (IgG) fraction and couple this fraction to activated Sepharose matrix as described previously (16). Final columns had a capacity higher than 1 μg for the compounds mentioned.

(h) *NaOH solution.*—1M.

(i) *Hydrochloric acid solution.*—0.01 and 1M.

(j) *Acetate buffer.*—2M (pH 5.2); 0.1M (pH 4.0).

(k) *β-Glucuronidase–sulfatase.*—Suc d'*Helix pomatia*, containing 100 000 units β-glucuronidase and 1 000 000 units sulfatase/mL (Industrie Biologique, France, code IBR 213473).

(l) *Solvents.*—Ethyl acetate, ethanol, toluene.

(m) *IAC eluting buffer.*—Ethanol–water–acetate buffer (0.1M, pH 4.0), (16 + 3 + 1, v/v/v)

(n) *Phosphate-buffered saline solution.*—0.02M, containing 0.02% thiomersal, pH 7.4.

(o) *Derivatization reagent.*—*N,O*-Bis(trimethylsilyl) trifluoracetamine (BSTFA) with 1% TMS (Cat. No. 38832, Pierce, Oud Beijerland, The Netherlands).

Apparatus

(a) *Gas chromatograph.*—HP Model 5890 (Hewlett Packard, Avoncale, PA), equipped with HP Model 7637A automatic injector, HP Model 5970 mass selective detector, HP Model 59970 workstation, and HP ink jet printer. Operating conditions: injection volume, 2 μL; injection temperature, 260°C; splitless injection mode; column temperature, 70°C, increase to 200°C at 25°C/min and hold 6 min, then increase to 300°C at 25°C/min and hold 2 min.

(b) *GC column.*—Fused silica permabond SE-52, 25 m × 0.25 mm id, film thickness 0.25 μm (Machery-Nagel, Düeren, Germany, No. 723054).

(c) *Digestion apparatus.*—Ultrasonic water bath, Bransonic 32 (Branson Europe, Soest, The Netherlands), or Subtilisin digestion (Sigma, P-5380).

(d) *Centrifuge.*—Sorvall RC-5B (Meyvis, Bergen op Zoom, The Netherlands).

(e) *Extrelut™ extraction cartridge.*—Merck, Darmstadt, Germany, Cat. No. 11737.

(f) *Rotary vacuum vaporizer.*—Rotavapor (Buchi, Mettler, Tiel, The Netherlands).

(g) *IAC columns.*—Pack with antibodies coupled to Tresyl-activated Sepharose™ (Pharmacia, Zoetermeer, The Netherlands). Store columns in phosphate buffered saline solution. After storage, prewash with at least 10 mL water.

Table 1. Standard compounds

Compound	CAS No.	Mol. formula	Mol. weight
Clenbuterol	37148-27-9	$C_{12}H_{18}Cl_2N_2O$	277.18
Salbutamol	18559-94-9	$C_{13}H_{21}NO_3$	239.31
Terbutaline	23031-25-6	$C_{12}H_{19}NO_3$	225.29
Cimaterol	54239-37-1	$C_{12}H_{17}N_3O$	219.29
Mabuterol	56341-08-1	$C_{13}H_{18}ClF_3N_2O$	310.75

Sample Preparation

Incurred urine samples were obtained by treating a cow over a 5-day period with 5 mg clenbuterol mixed into the feed supply each day. Another animal was treated according to the same regimen with 10 mg salbutamol. Samples of pig liver were obtained by treatment of a single pig with 2 mg clenbuterol and 4 mg salbutamol per day over a 31-day period. Samples for quality assurance were prepared by diluting incurred materials with materials obtained from control animals known to be free of any β-agonist treatment. Animal samples were stored at −20°C until analysis.

Extraction Procedures

For the extraction of liver, several procedures are possible, e.g., subtilisin digestion (19) or ultrasonic methods. Studies at RIVM revealed no significant analytical difference in analytical results when extractants from either procedure were used. However, the ultrasonic procedure is easier to use and results in fewer interfering coextractives. In general, ultrasonic extraction in dilute acid is preferred (20).

Animal Feed

Accurately weigh a test portion of 10.0 g powdered animal feed into a glass flask and add 50.0 mL water and internal standards (maximum volume 0.5 mL). Place flask in ultrasonic water bath and extract 30 min, shaking at least every 10 min. Subsequently shake flask 10 s by hand. Centrifuge flask 10 min (40 000 × *g*) and pipet 19 mL into clean flask. Adjust pH to 9.8 ± 0.2 with 1M NaOH or 1M HCl. If necessary, use smaller test portions, e.g., in the case of medicated feed premixes or their constituents.

Liver

Accurately weigh a test portion of 3.0 g homogenized liver into 20 mL glass flask; add 9 ng each internal standard and 15 mL 0.01M HCl. Place flask in ultrasonic water bath and extract 15 min. Shake or vortex flask 10 s. Centrifuge flask 10 min (1800 × *g*) and decant supernatant into clean flask. Adjust pH to 5.2 ± 0.1 with 1M NaOH.

Urine

Accurately pipet a test portion of 10.0 mL urine into 20 mL glass flask and add 30 ng each internal standard. Adjust pH to 5.2 ± 0.1 with 1M NaOH or 1M HCl.

Enzymatic Deconjugation

To test portions of urine or liver extract, add 2 mL acetate buffer (2M) and 0.1 mL β-glucuronidase–sulfatase. Incubate overnight (18 h) at 37°C. Cool incubates to room temperature and adjust volume to 20 mL with water.

Extrelut Extraction

Place 20 mL aqueous extract or sample onto Extrelut column. Equilibrate 15 min, then extract absorbed aqueous phase with 60 mL ethyl acetate and evaporate eluate on rotary vacuum vaporizer at 50°C.

Sample Cleanup and Immunoaffinity Chromatography

Prepare and characterize IAC materials according to standardized procedures (21). In short, isolate IgG fraction from rabbit antiserum by IAC on protein A Sepharose. Measure protein and couple IgG to one of the activated matrixes. To estimate percent IgG coupled to matrix, measure protein content again after coupling. Evaluate capacity of gel, and, if adequate, fill individual columns.

Dissolve dry residue of ethyl acetate eluate containing analytes in 0.1 mL ethanol. Add 50 mL water and immerse flask in ultrasonic water bath for at least 1 min. Apply aqueous extract to IAC column and percolate at 2 mL/min. Wash column with 5 mL water and elute β-agonists with 5 mL IAC eluting buffer. Evaporate eluate to dryness in water bath at 50°C under cold stream of nitrogen. Regenerate IAC column by subsequent washings with 10 mL IAC eluting buffer, 25 mL water, and 25 mL phosphate-buffered saline solution. Store column wet at 4°C in this buffer.

Derivatization

Dissolve dry residue of IAC eluate in 0.1 mL derivatization reagent. Incubate reaction mixture 1 h at 60°C. Evaporate reagent and dissolve dry derivatized residue in 0.05 mL toluene. Transfer toluene solution to injection vial and perform GC/MS analysis.

GC/MS Analysis

The first GC/MS run is for screening purposes only. Therefore, only the following ions are monitored: m/z = 86 (*tert*-butyl-β-agonists); m/z = 72 (isopropyl-β-agonists); and m/z = 92 (d_6-internal standards).

Under the conditions specified, the compounds mentioned have retention times ranging from ca 9–15 min. The procedure used for quantitation depends on the internal or external standards used. When isotope-labeled standards are used, a linear calibration curve can be fitted with the ID-ratio (ratio of abundances of ions m/z 86 and m/z 92) as the independent variable and the concentration of standard (e.g., ng/injection vial) as the dependent variable. This procedure yields linear calibration curves with an intercept not significantly different from zero.

For other β-agonists, quantitation is less straightforward. In practice, however, recovery for most compounds is equal to the recovery of clenbuterol, both for muscle and liver. The only compound with significantly different analytical recovery is salbutamol. For this compound, however, an internal standard is available (see *Results*).

Confirmation of Identity

For purposes of confirmation of the identity, additional (fragment) ions must be monitored. TMS derivatives of the appropriate β-agonists fragment according to the pattern shown in Figure 3. According to EC criteria for reference methods, identification of compounds by low-resolution MS must be based on at least 4 diagnostic ions that all elute simultaneously from the GC column; all must have a response signif-

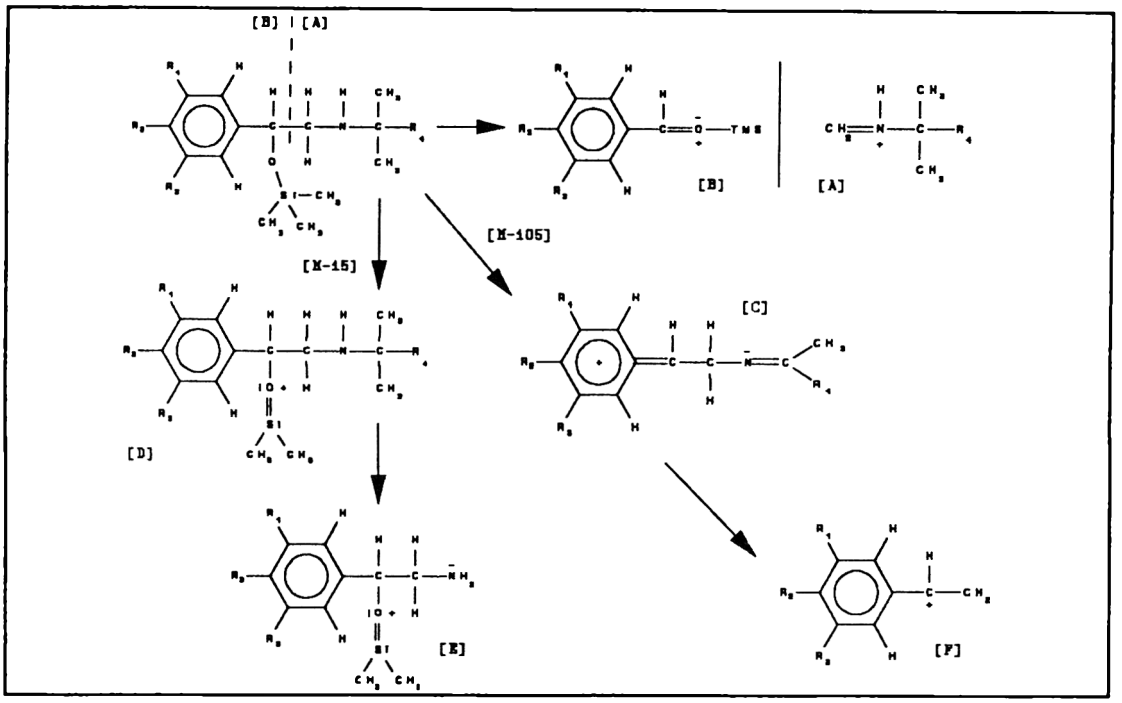

Figure 3. Fragmentation pattern of β-agonists.

icantly higher than the average noise. Moreover, the response ratios must be in agreement with the corresponding ratios for standard compounds. The masses of the ions [A]–[F] for the 5 most important β-agonists are summarized in Table 2. Figure 4 shows the mass spectrum of clenbuterol. The major response is observed for ion m/z = 86; all other ions show only very limited abundances.

Results

A number of studies were undertaken to validate the analytical procedure. Most of these experiments were intralaboratory experiments (repeatability and within-laboratory reproducibility). In addition, the method was validated in a cooperative interlaboratory study on animal feed, organized by the Community Reference Bureau (BCR) of the EC and demonstrated during an EC workshop in The Netherlands.

Analytical Recovery During IAC

Each newly prepared batch of IAC material must be tested for its capacity to retain relevant components. For a representative batch, the average recovery at the 200 ng level was 101 ± 5% (mean ± standard deviation, N = 8). No differences were observed between the different β-agonists tested.

Analytical Recovery of the Procedure

When the IAC materials were demonstrated to be suitable, the procedure was validated by determining the analytical recovery of analytes spiked to blank urine. Results are summarized in Table 3. During these experiments, the internal standard was not added in the beginning of the procedure but after the chromatography steps. Corrections were made only for differences during derivatization and GC/MS analysis and not for losses during extraction and extract cleanup; therefore, recoveries of the extraction and cleanup steps were obtained.

Similar experiments were performed with blank liver spiked with 5 μg/kg of the above-mentioned β-agonists. As with most analytical procedures, recovery from tissue is significantly less than from urine. For salbutamol, recovery was in the range of 40–50% (N = 5); for all other compounds, it was 65 ± 8% (N = 6).

To test for the effectiveness and necessity of enzymatic hydrolysis, urine samples from animals treated with clenbuterol or salbutamol were analyzed under different conditions. For clenbuterol, no significant effects were observed, indicating stability during prolonged incubations at an elevated temperature and the absence of glucuronide and/or sulfate conjugates. For salbutamol, however, dramatic differences were

Table 2. Masses of diagnostic ions after EI-ionization of β-agonists–TMS derivatives

Compound	N[a]	M_0[b]	M_d[c]	Ions [A]	[B]	[C]	[D]	[E]	[F]
Terbutaline	3	225	441	86	356	336	426	370	280
Salbutamol	3	239	455	86	369	350	440	384	294
Clenbuterol	1	276	348	86	262	243	333	277	187
Cimaterol	1	219	291	72	219	186	276	234	—
	2	219	363	72	291	258	348	—	—
Mabuterol	1	310	382	86	296	277	367	311	221

[a] N = number of TMS groups.
[b] M_0 = molecular mass.
[c] M_d = molecular mass after derivatization.

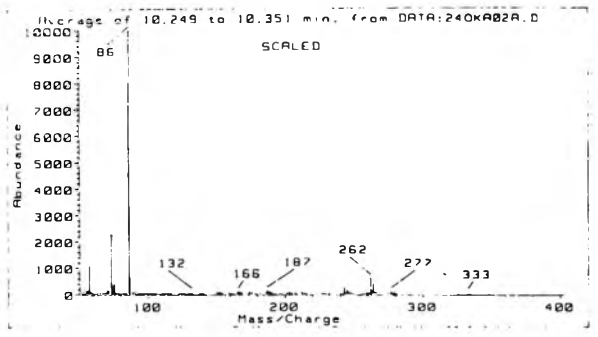

Figure 4. Mass spectrum (EI ionization) of clenbuterol–TMS.

observed. Figure 5 [logarithm of the ratio C_t/C_0, where C_t = concentration at time t and C_0 = concentration at t = 0 (4 μg/L)] summarizes the results of an experiment in which a sample of urine (cattle) was incubated for selected times between 0 and 18 h. The salbutamol concentration increased during this time from 4 to 134 μg/L, indicating almost complete conjugation. Figure 5 shows a smooth curve from which the final concentration is estimated to be approximately 145 μg/L. The straight line applicable for incubation times up to 6 h indicates first-order kinetics during this period. On the basis of these results, we decided that an overnight enzymatic incubation is routinely necessary, apart from cases of confirmation specific for clenbuterol. Because no data are available for compounds other than clenbuterol and salbutamol, and only limited data are available for other species, an enzyme preparation containing both glucuronidase and sulfatase activity was selected.

Repeatability and Reproducibility

Repeatability and within-laboratory reproducibility were determined for clenbuterol and salbutamol by analyzing samples of urine and liver obtained from treated animals (urine from bovine and liver from a pig). Samples were analyzed on 3 different occasions, each time in duplicate (Table 4).

Cooperative Study on Animal Feed

During 1990, the method was validated during a cooperative study on clenbuterol in animal feed, organized by the EC. Thirteen laboratories participated and 4 different samples were analyzed 4 times. The within-laboratory variability (% RSD) was <10% for all samples. The average value obtained with this method was always within ±10% of the target value and never significantly different from the overall average value.

EC Workshop

The analytical procedure described was the subject of an official EC Workshop organized at our institute (22). Attention was focused on the analyses of urine (bovine) for clenbuterol and salbutamol. Participants originating from 12 different European countries analyzed samples without any previous experience with the method. For clenbuterol, the result was 5.7 ± 0.2 μg/L (mean ± SD, N = 6), for salbutamol, 3.6 ± 0.4 μg/L (mean ± SD, N = 6). The identification criteria was fulfilled for each analysis.

Table 3. Analytical recovery from pig urine

Compound	Analytical rec., % (av. ± SD, N = 4)
Clenbuterol	103 ± 9
Salbutamol	58 ± 6
Mabuterol	112 ± 5
Cimaterol	99 ± 6

Discussion

Laboratories involved in residue analysis of veterinary drugs and growth-promoting agents must analyze a variety of compounds in biological samples, black market preparations, and animal feed. Within our laboratory, therefore, we focused on the development of multimatrix and multianalyte methods. Earlier studies demonstrated the suitability of IAC for sample cleanup for single compounds (16) and in multiresidue procedures (17). In these latter methods, cocktails of IgG fractions coupled to an activated matrix were used, which differs from the approach used here.

Only 2 different antibodies were used to prepare an IAC column suitable for a large number of compounds. The reason is obvious: By coupling clenbuterol and cimaterol through their aromatic amine function to BSA, without incorporating an additional bridging group as spacer, antibodies were primarily raised against the *N-tert*-butyl and *N*-isopropyl-ethanolamino moieties of the molecules. Most β-agonists known to be illegally used in meat production contain either one of these 2 groups. The relative nonspecificity of these antibodies does not influence the final result because identification is not based on the interaction with the antibodies but on at least 4 ions monitored during GC/MS analysis.

Some important aspects of methods for residue analysis are the limits for detection, determination, and, most of all, identification. During initial screening, only the ions with m/z = 72 or 86 are monitored. Because these ions contain most of the intensity of the total response, very sensitive detection is allowed: i.e., a limit of determination of 0.2 μg/L and a limit of detection, defined as the average noise + 3 SD, in the range of 0.05–0.1 μg/L for urine. However, although the fragmentation of β-agonists may be advantageous for screening, it is highly disadvantageous for identification because 4 ions now have to be monitored. In practice, the limit of identification becomes equal to the limit of detection for the weakest ion within the set of 4 ions selected. Figure 6 shows 2 of the 4 ion traces of a sample of urine containing approximately 1 μg/L clenbuterol. The lower panel shows the response for ion m/z = 86, the most abundant ion on which the limit of detection is based; the upper panel shows the ion with the lowest abundance (m/z = 333), which defines the limit of identification.

Therefore, for clenbuterol, the β-agonist with the poorest spectrum for identification, the limit of identification is within

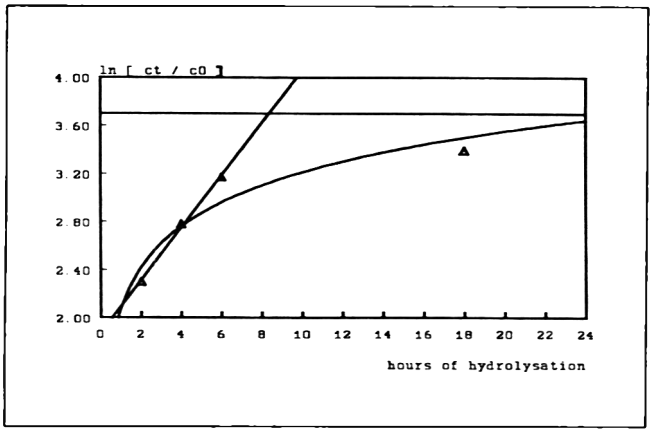

Figure 5. Salbutamol concentration after different incubation times with *Helix pomatia*.

Table 4. Within- and between-assay variability

Individual values, ppb		Within-assay	Between-assay
		Salbutamol in bovine liver	
3.6	3.3	s^2 = 0.016	s^2 = 0.065
3.4	3.3	s = 0.13	s = 0.25
3.7	3.7	% RSD = 3.7	% RSD = 7.1
		Clenbuterol in bovine liver	
13.8	11.2	s^2 = 1.31	s^2 = 2.63
12.9	12.6	s = 1.14	s = 1.62
15.1	14.1	% RSD = 8.5	% RSD = 12.1
		Clenbuterol in pig urine	
4.4	4.3	s^2 = 0.035	s^2 = 0.122
4.2	4.6	s = 0.19	s = 0.35
4.7	4.9	% RSD = 4.2	% RSD = 7.8
		Salbutamol in pig urine	
2.0	2.0	s^2 = 0.007	s^2 = 0.020
1.8	2.0	s = 0.08	s = 0.14
2.1	2.1	% RSD = 4.0	% RSD = 7.0

the 1–2 μg/L range, which in some cases might be insufficient. The use of chemical ionization techniques, however, can circumvent these problems. As a practical rule, however, electron impact ionization in combination with the analytical procedure described can cope with the identification of relevant analytes in most samples.

Up to now, most residue data for animals treated under controlled conditions are available for clenbuterol (9) and salbutamol (10). Half-life values for residues in urine are all in the range of 1–3 days, allowing detection of residues up to 2–3 weeks after treatment.

In the procedure used, a deconjugation step is routinely applied. For clenbuterol, no data are available that suggest that conjugation occurs. Also in our studies, no effect of enzymatic hydrolysis on the measured clenbuterol concentration was observed. For salbutamol, published data indicate that in humans the 4′-*O*-sulfate ester of salbutamol is the major fraction of the total amount present (23–26). Detailed studies showed that enzymatic hydrolysis with *Helix pomatia* preparations is a suitable procedure for liberating salbutamol (25). Our results are in good agreement with these studies; the percentage conjugated salbutamol in cattle can be close to 100%. Detailed studies on the hydrolysis of salbutamol-4′-*O*-sulfate ester, however, were not possible because the pure compound is not available. Also, the presence of salbutamol–glucuronide has been described in dogs and rabbits (25).

The procedure has mainly been tested for clenbuterol, cimaterol, salbutamol, terbutaline, and mabuterol, but other β-agonists containing an *N*-*tert*-butyl or *N*-isopropyl group, such as isoprenaline, carbuterol, and pirbuterol, can be analyzed with this procedure. During surveillance studies within The Netherlands so far, only clenbuterol, salbutamol, and, to a lesser extent, mabuterol have been detected in samples of liver and urine. Therefore, most of our attention during validation was focused on the first 2 compounds. Within-assay variability (repeatability) and between-assay variability (within-laboratory repeatability) were determined for both compounds in liver and urine and were expressed as % RSD. These variabilities were always better than 15%, well below the EC limits for reference methods. Despite recent developments in the analysis for β-agonists, reproducibility studies have not been performed because, within the EC, only a limited number of laboratories are involved.

The EC Bureau of Reference (BCR) organized a cooperative study on clenbuterol in animal feed using the method described here; results were excellent. The method was used successfully at an EC workshop for determining residues of clenbuterol and salbutamol in urine, and a collaborative study of the method is being organized within the EC. Already, 30 laboratories have expressed an interest in participating.

The combination of IAC with GC/MS in multiresidue analyses for veterinary drugs, including growth promoters, is a frequently used analytical strategy within our laboratory. In procedures for β-agonists, this strategy has been particularly useful because, with a combination of only 2 antibodies, a large range of compounds can be detected and analyzed. An additional advantage is the possibility of monitoring for compounds not detected before, provided they have a *tert*-butyl or an isopropyl group. Several "new" β-agonists were detected in premixes and finished animal feed.

The identification of such "unknown" β-agonists is one of our continuing projects. By use of IAC, GC/MS, FTIR, and NMR, 3 such compounds have been identified recently in our institute as the *N*-*tert*-butyl analogue of cimaterol and its corresponding bromo intermediate, and as the *N*-pentyl analogue of mabuterol (26, 27). According to its MS fragmentation pattern, the pentyl group of the latter compound is tentatively identified as a *tert*-pentyl group (28). Two of these compounds were also found during surveys in Belgium (29).

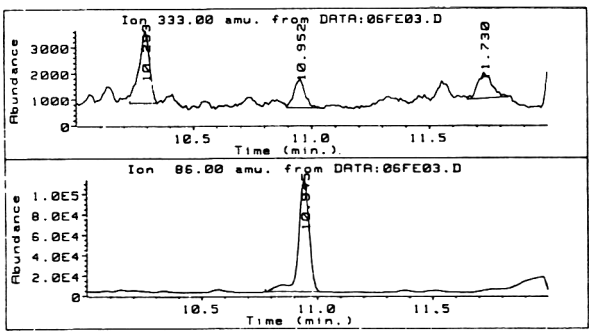

Figure 6. Confirmation of clenbuterol. Two of the 4 ion traces are shown: upper panel, ion with lowest abundance; lower panel, ion with greatest abundance.

For IAC to become a routine analytical technique within a large group of laboratories, IAC materials must be readily available. By now, several commercial companies are active in this area, supplying single- and multianalyte columns. We believe that IAC in combination with GC/MS (low resolution) is a reliable and low-cost analytical tool, suitable for both screening and confirmation of a variety of compounds, β-agonists in particular.

Acknowledgments

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VITAMINS

Liquid Chromatographic Determination of Thiamine, Riboflavin, and Pyridoxine in Infant Formula

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An ion pairing reversed-phase liquid chromatographic method developed for multivitamin supplements and premixes was applied to the simultaneous determination of thiamine, riboflavin, and pyridoxine in perchloric acid extracts of milk- and soy-based infant formulas. The method uses *m*-hydroxybenzoic acid as internal standard and a mobile phase consisting of water, acetonitrile, hexanesulfonic acid sodium salt, and ammonium hydroxide solution, adjusted to pH 3.6 with phosphoric acid. The column is a 15 cm × 3.9 mm id Nova Pak C18. Limits of detection were 0.15 μg/mL for thiamine and 0.09 μg/mL for riboflavin by UV detection at 254 nm, and 0.010 μg/mL for pyridoxine by fluorescence detection. Mean percent recoveries based on triplicate determinations were 102 ± 1.8, 102 ± 3.3, and 101 ± 3.1 for thiamine, riboflavin, and pyridoxine, respectively. The results compared favorably with the AOAC methods for thiamine, riboflavin, and pyridoxine.

Current AOAC methods for the determination of thiamine (B_1), riboflavin (B_2), and pyridoxine (B_6) use chemical and microbiological techniques. A simultaneous liquid chromatographic (LC) assay for these vitamins in infant formula would be desirable from an analytical and economic standpoint. We recently published a reliable simultaneous determination of B_1, B_2, B_6, and niacin in multivitamin premixes and tablets (1). To apply the simultaneous determination to infant formula, an extraction procedure compatible with the LC system was needed to extract the vitamins from the formula matrix.

Several types of extractions were developed for use in conjunction with LC determination of water-soluble vitamins in food products. Extraction procedures used by Wehling and Wetzel (2) required fairly cumbersome treatments for B_1, B_2, and B_6. Wehling and Wetzel (2) extracted samples with 0.1N sulfuric acid in a boiling water bath, digested the extracts by a fungal amylase preparation, and then centrifuged and filtered the digestates. Rees (3) developed an extraction procedure for B_6 and nicotinamide in fortified foods by using 1M sulfuric acid treatment in a boiling water bath. Although these extractions are fairly straightforward, we have had difficulty in obtaining good chromatograms for infant formula with extracts that were heat-treated.

With regard to B_6, Vanderslice et al. (4) stated that "early extraction procedures using HCl in conjunction with autoclaving have generally been replaced by procedures that use trichloroacetic, perchloric, tungstic, metaphosphoric, or sulfosalicylic acids" before LC analysis. Vanderslice et al. (4) further indicated that the extraction procedure must be compatible with the final analytical procedure to yield extracts that are clean and free from interference. Vanderslice et al. (5) and Gregory and Feldstein (6) used sulfosalicylic acid (SSA) to extract B_6. In later work, Vanderslice and Huang (7) used SSA to extract B_1 and a cleanup column to remove SSA, which fluoresces strongly and can interfere with the analysis.

Pierotti et al. (8) used perchloric acid extraction for the determination of B_6 in rat tissues. After treatment, the acid was precipitated with 6M potassium hydroxide. For B_6 derivatives in foods, Toukairin-Oda et al. (9) also used perchloric acid extractions followed by potassium hydroxide precipitation at pH 3.5 and added a step in which the sample was left overnight to completely precipitate the perchloric acid. Of the various extractions, the perchloric acid extraction seems the most compatible with the chromatographic conditions described by Chase and Soliman (1). After the perchloric acid was precipitated, the pH would be close to that of the mobile phase used in this study.

In the present study, the LC procedure of Chase and Soliman (1) was applied to the simultaneous determination of B_1, B_2, and B_6 in infant formula with perchloric acid as the extractant.

METHOD

Reagents

(a) *Perchloric acid.*—70% double distilled (G. Frederick Smith Chemical Co., Columbus, OH 23214).

(b) *Acetonitrile.*—Distilled-in-glass UV grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442).

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(c) *Hydrochloric acid.*—Reagent grade (Fisher Scientific Co., West Haven, CT 06516).

(d) *Phosphoric acid.*—Reagent grade (Fisher Scientific Co.).

(e) *Ammonium hydroxide.*—Reagent grade (Fisher Scientific Co.).

(f) *Potassium hydroxide.*—Reagent grade (Fisher Scientific Co.).

(g) *Ion-pairing reagent.*—1-Hexanesulfonic acid sodium salt (Eastman Kodak Co., Rochester, NY 14650).

(h) *Internal standard.*—*m*-Hydroxybenzoic acid (*m*-HBA) (Sigma Chemical Co., St. Louis, MO 63178).

(i) *Standard solutions.*—Individual stock standards of thiamine HCl (0.1 mg/mL), riboflavin (0.02 mg/mL), and pyridoxine HCl (0.1 mg/mL or 0.08 mg/mL as free base) were prepared from USP Standards (Rockville, MD 20852) in 0.1N HCl. Stock standards were stable for several months under refrigeration.

(j) *Internal standard solution.*—Stock internal standard was prepared by dissolving 55 mg *m*-HBA in 100 mL mobile phase (550 μg/mL).

(k) *Mobile phase.*—1-Hexanesulfonic acid (0.95 g) was dissolved in 1 L water containing 9.5% acetonitrile and 0.5 mL ammonium hydroxide. Solution was adjusted to pH 3.60 with phosphoric acid. A 950 mL portion of this solution was diluted to 1 L with water. Mobile phase was then filtered through 0.45 μm nylon filter (Universal Scientific, Inc., Atlanta, GA 30360).

Apparatus

(a) *Liquid chromatograph.*—Model HP1090 (Hewlett-Packard Co., Palo Alto, CA 94303) equipped with filter photometric detector, Model 85B computer, and Model 3392A integrator.

(b) *Column.*—Nova Pak C18 stainless steel, 15 cm × 3.9 mm id, No. 0863440, with an in-line precolumn filter, No. 84560 (Waters Associates, Milford, MA 01757).

(c) *Fluorescence detector.*—Perkin-Elmer (Norwalk, CT 06859) Model 650-105 equipped with Model 650-8001 microflow cell unit, and Hewlett-Packard Model 3390A integrator.

(d) *Absorbance Detector.*—Model 440 UV/VIS (Waters Associates).

Chromatographic Conditions

Instrument parameters.—Injection volume 50 μL (Rheodyne Loop Injector Model No. 7010, Rheodyne, Inc., Cotati, CA 94928); flow rate 1.0 mL/min; UV absorbance detector wavelength 254 nm; parameters for fluorescence detection (in series with UV detector) included sensitivity 0.3, excitation wavelength 295 nm, slit 7 nm, emission wavelength 395 nm, and slit 7 nm.

Sample Description and Preparation

Infant formula samples used in this study consisted of 2 powders, 1 ready-to-feed, and 6 concentrates. Samples also represented different formulation bases and consisted of 4 soy-based formulas, 3 whey/milk-based formulas, 1 milk/whey-based formula, and 1 milk-based formula. These samples represent formulas produced to meet nutrition requirements for normal infants as specified by Infant Formula Act of 1980 (P.L. 96–359). Sample preparations were performed under subdued light, according to AOAC instructions (10) for proper warming, opening, mixing, and storage under nitrogen atmosphere and refrigeration.

Sample Extraction

Powder.—Accurately weigh ca 12 g powder into 250 mL Philips beaker and disperse in 50 mL water. Stir mixture until homogenous.

Ready-to-feed.—Accurately weigh ca 70 g liquid into 250 mL Philips beaker.

Concentrates.—Accurately weigh ca 35 g liquid into 250 mL Philips beaker and mix with 20 mL water.

Add 2 mL perchloric acid to each solution with stirring, and continue stirring 1 h. Carefully add 6M potassium hydroxide dropwise with constant stirring until pH is 3.3 ± 0.3. Transfer each solution quantitatively to 100 mL volumetric flask and dilute to volume with mobile phase. Refrigerate solutions overnight to allow perchlorate to precipitate completely and then filter through 32 cm grade 588 prepleated filter paper (Schleicher and Schuell, Keene, NH 03431). Add 100 μL internal standard to 10 mL volumetric flask and dilute to volume with sample solution. Filter solutions through 0.45 μm nylon filter (Universal Scientific, Inc.).

Standard Extraction

Prepare working standard by combining 2.0, 10.0, and 2.0 mL aliquots of B_1, B_2, and B_6, respectively, in 250 mL Philips beaker containing ca 40 mL water. From this point on, treat standard and sample the same, beginning with "add 2 mL perchloric acid...," except transfer 5.0 mL from final filtered standard to 10 mL volumetric flask containing 100 μL *m*-HBA and dilute to volume with mobile phase to give final concentration of 1.0, 1.0, 0.82, and 5.5 μg B_1, B_2, B_6, and *m*-HBA/mL, respectively.

System Calibration and Assay

Establish peak response and retention time parameters by injecting working standard in duplicate. Enter these parameters, along with standard concentrations (μg/mL), into integrator's internal standard calibration table to calculate levels of B_1, B_2, and B_6. Establish linearity by running series of 4 standard dilutions for each vitamin in duplicate, ranging in concentration from 0.2 to 1.4 μg/mL for B_1 and B_2 and from 0.2 to 1.2 μg/mL for B_6.

Calculations

The integrator prints out concentrations of each vitamin assayed as μg/mL. Concentration (μg of each vitamin per 100 g of ready-to-feed) was calculated for powders as follows:

$$\mu g \text{ vitamin}/100 \text{ g as ready-to-feed} = (\mu g/mL \times DF \times F \times 100) / SW$$

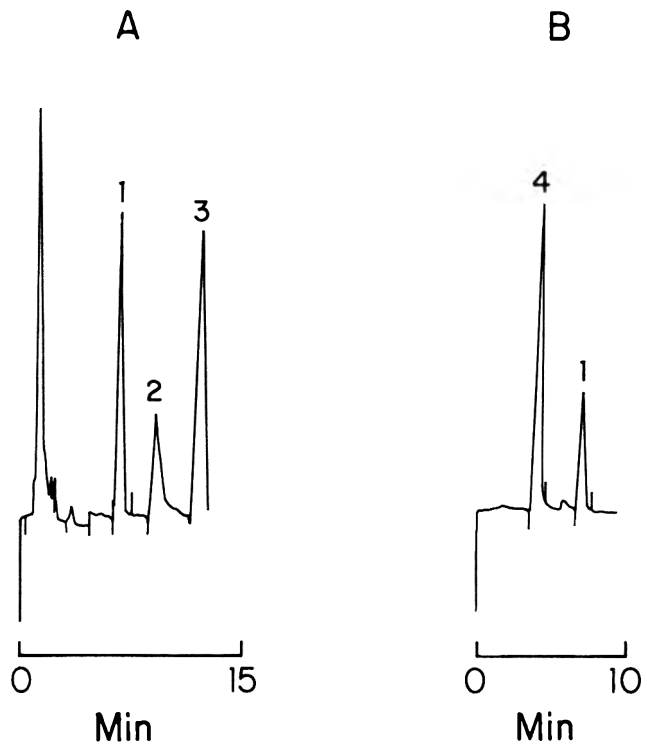

Figure 1. Chromatogram of working standard mixture by (A) UV detection (254 nm) and (B) fluorescence detection (excitation wavelength, 295 nm; emission wavelength, 395 nm): (1) internal standard, (2) thiamine, (3) riboflavin, and (4) pyridoxine.

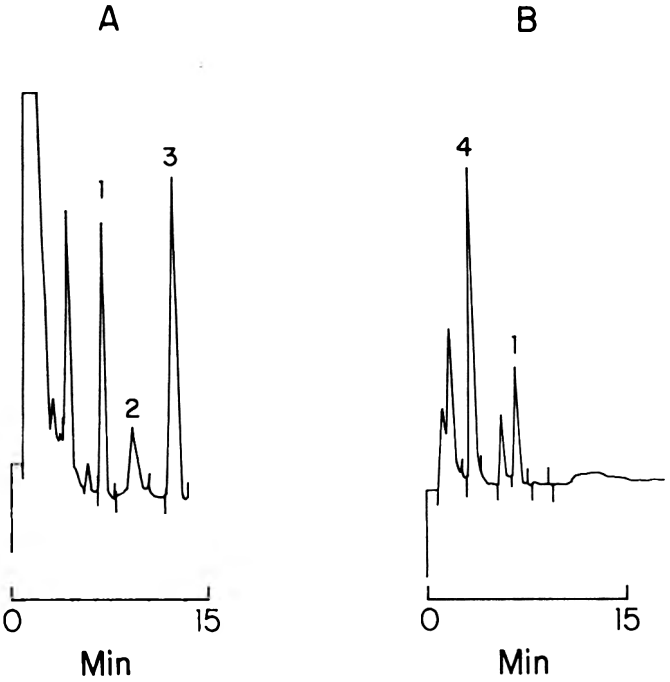

Figure 2. Chromatogram of milk-based infant formula extract by (A) UV detection (254 nm) and (B) fluorescence detection (excitation wavelength, 295 nm; emission wavelength, 395 nm): (1) internal standard, (2) thiamine, (3) riboflavin, and (4) pyridoxine.

For ready-to-feed:

$$\mu g \text{ vitamin}/100 \text{ g} = (\mu g/mL \times DF \times 100)/SW$$

For concentrates:

$$\mu g \text{ vitamin}/100 \text{ g as ready-to-feed} = (\mu g/mL \times DF \times 100)/(SW \times 2)$$

where DF = dilution factor; SW = sample weight, g; and f = powder weight, g, of reconstituted infant formula.

AOAC Methods Used for Comparison

The following AOAC methods were used for analysis of infant formulas: B_1, manual thiochrome method (11); B_2, manual fluorometric method (12); B_2, automated fluorometric method (13); B_2, microbiological method (14); and B_6, microbiological method, except that column chromatography to separate B_6 vitamins was not used (15).

Results and Discussion

Figures 1 and 2 show that B_1, B_2, B_6, and *m*-HBA are well-resolved and each vitamin can be simultaneously determined in an infant formula extract. The chromatogram of the working standard mixture by UV detection is illustrated in Figure 1A. Excellent peak responses were obtained for *m*-HBA, B_1, and B_2. Figure 1B illustrates detection of standard B_6 and *m*-HBA by fluorescence.

Figure 2A depicts the elution profile of a milk-based infant formula extract for B_1 and B_2 by UV. Figure 2B is the elution profile of the same extract obtained by fluorescence detection for B_6. The B_6 peak is well-resolved and free of chromatographic interferences present in the UV profile. For milk-based formulas, 15 min was required for eluting all peaks and returning to base line before the next injection. However, 45 min was required for soy-based formulas to allow for late-eluting peaks before returning to base line.

As noted in the earlier work by Chase and Soliman (1), the standard UV response was linear from 0.2 to 1.4 μg/mL for B_1 and B_2 and from 0.2 to 1.2 μg/mL for B_6. The limit of detection was 0.2 μg/mL for each vitamin with UV detection. In this study, when a Waters 440 spectrophotometric detector was substituted for the Hewlett-Packard filter photometric detector, detection limits improved to 0.15 μg B_1/mL and 0.09 μg B_2/mL. The use of a fluorescence detector increased the detection level for B_6 to 0.01 μg/mL. Three infant formula samples (2 milk-based and 1 soy-based) were used for recovery studies. The mean percent recoveries (n = 3) and standard deviations were 102 ± 1.8, 102 ± 3.3, and 101 ± 3.1 for B_1, B_2, and B_6, respectively. The system reproducibility was evaluated by performing 10 repetitive analyses of an infant formula powder labeled to contain 52.8, 106, and 41.9 μg of B_1, B_2, and B_6/100 g, respectively. The mean, standard deviation, and coefficient of variation were 60.8 ± 0.5 (CV 0.9%), 151 ± 2.1 (CV 1.4%), and 39.4 ± 0.7 (CV 1.8%) for B_1, B_2, and B_6, respectively.

The LC results were further compared with those obtained by AOAC methods for the determination of such vita-

Table 1. Comparison of values (μg/100 g) obtained for thiamine in infant formula by LC and AOAC methods

Infant formula	Declared	LC method[a]	AOAC manual[b]
Whey/milk powder	52.8	65.2 ± 4.8	78.4 ± 0.9
Whey/milk ready-to-feed	51.7	70.5 ± 3.0	51.7 ± 1.9
Whey/milk concentrate	50.0	63.5 ± 7.8	59.5 ± 4.0
Soy concentrate	38.5	106 ± 2.6	91.5 ± 0.1
Milk concentrate	64.2	112 ± 2.1	116 ± 1.4
Soy concentrate	60.4	46.6 ± 2.4	48.1 ± 1.1
Soy concentrate	64.1	63.3 ± 0.7	75.5 ± 0.1
Milk/whey concentrate	64.4	93.5 ± 6.1	91.5 ± 0.3
Soy powder	67.6	88.4 ± 5.5	89.4 ± 1.7

[a] LC data are means of triplicate injections for duplicate extractions.
[b] AOAC data are averages of duplicate extractions.

mins. Table 1 illustrates the declared amounts of B_1 and the results obtained by the LC method and the AOAC manual thiochrome method (11). A comparison of the LC method with the AOAC method gave a mean (n = 9) ratio of 1.03, which indicates close agreement between methods. (An ideal ratio of the results of the LC method to the AOAC method would be 1.00.) A linear regression analysis comparing the LC method to the AOAC method, where $y = mX + b$, gave a correlation coefficient of r = 0.951, where r =

$$\frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{n(\sum x^2) - (\sum x)^2}\sqrt{n(\sum y^2) - (\sum y)^2}}$$

The fact that both the LC and AOAC results are higher than the declared values (as calculated on a μg/100 g ready-to-feed basis) is due to overages added by the manufacturers to compensate for potential losses during production and storage of the product, as discussed by Martin et al. (16). Similar overages are observed in the values of B_2 and B_6 determination by LC and AOAC methods.

Table 2 illustrates the declared amounts of B_2 and the results obtained by the LC method, the AOAC manual fluorometric method (12), the AOAC automated fluorometric method (13), and the AOAC microbiological method (14). A comparison of the LC and manual fluorometric results gave a mean ratio (n = 9) of 0.73 (r = 0.968). The higher B_2 levels obtained by the AOAC manual fluorometric method can be attributed to extraneous fluorescent compounds in the sample matrix. This is supported by Egberg and Potter (17), who observed that the AOAC manual fluorometric method (12) gives higher results for certain products containing impurities, which can be eliminated by the use of the Florisil column cleanup step.

This difference between the LC method and the AOAC manual fluorometric method was of great concern because the manual fluorometric method is the official AOAC method for B_2 determination in milk-based infant formulas. Accordingly, this problem was further pursued by comparing LC results with those obtained by the AOAC automated and microbiological methods. Results in Table 2 show close agreement between the LC method and the automated fluorometric method with a mean ratio (n = 9) of 0.94 (r = 0.979). This agreement is most probably due to the use of dialysis membranes to remove sample matrix interferences before the determinative step. Further comparison of the LC method to the microbiological method gave a mean ratio (n = 9) of 1.13 (r = 0.978), indicating that the results of the microbiological assay compare favorably with those by the LC method and are within acceptable limits of variation.

Table 3 illustrates the declared amounts of B_6 and the results obtained by the LC method and the AOAC microbiological method. The LC method, which measures only the added pyridoxine form, gave results exceeding the declared values of fortified B_6 in 89% of the samples studied. Comparison of the LC results to those of the microbiological assay gave a mean ratio (n = 9) of 0.69 (r = 0.976). The higher values obtained by the microbiological assay are most probably due to the thermal hydrolysis used in the microbiological assay to free the bound B_6 forms. In a review by Gregory (18), acidic digestion coupled with 2–5 h of autoclaving is necessary to free the bound B_6 from protein, glucoside conjugates, and phosphate esters because bound B_6 forms cannot be used by the microorganisms to show growth response. Accordingly, the perchloric acid digestion in this procedure only extracts the added B_6 form.

Table 2. Comparison of values (μg/100 g) obtained for riboflavin in infant formula by LC and 3 different AOAC methods

Infant formula	Declared	LC method[a]	AOAC manual[b]	AOAC automated[b]	AOAC microbiological[b]
Whey/milk powder	106	141 ± 2.7	228 ± 13.4	141 ± 2.8	132 ± 5.7
Whey/milk ready-to-feed	103	143 ± 2.1	178 ± 22.6	134 ± 14.1	131 ± 10.3
Whey/milk concentrate	100	142 ± 1.4	180 ± 23.3	147 ± 0	131 ± 8.7
Soy concentrate	57.8	72.5 ± 3.0	98.0 ± 0.8	76.0 ± 2.3	61.7 ± 3.9
Milk concentrate	96.4	152 ± 3.9	216 ± 10.6	165 ± 5.0	117 ± 7.0
Soy concentrate	60.4	99.0 ± 9.6	147 ± 2.8	113 ± 5.7	78.0 ± 8.2
Soy concentrate	96.1	142 ± 5.9	180 ± 5.0	154 ± 7.1	116 ± 4.6
Milk/whey concentrate	96.6	180 ± 2.1	280 ± 7.1	217 ± 2.1	181 ± 12.1
Soy powder	101	166 ± 9.3	211 ± 5.0	165 ± 0.7	145 ± 5.6

[a] LC data are means of triplicate injections for duplicate extractions.
[b] AOAC data are averages of duplicate extractions.

Table 3. Comparison of values (μg/100 g) obtained for B_6 in infant formula by LC and AOAC methods

Infant formula	Declared	LC method[a]	AOAC microbiological[b]
Whey/milk powder	41.9	43.0 ± 0.7	72.3 ± 3.5
Whey/milk ready-to-feed	41.1	44.8 ± 1.4	60.2 ± 0.5
Whey/milk concentrate	39.8	43.2 ± 1.1	59.5
Soy concentrate	38.5	43.4 ± 0.5	68.0 ± 0
Milk concentrate	38.5	41.3 ± 0.2	56.5
Soy concentrate	55.2	41.4 ± 0.6	62.9
Soy concentrate	40.1	53.5 ± 0.5	77.5
Milk/whey concentrate	40.2	41.5 ± 0.7	64.5
Soy powder	42.3	86.4 ± 2.4	110 ± 1.4

[a] LC data are means of triplicate injections for duplicate extractions.
[b] AOAC data with standard deviations are means of duplicate determinations.

Peak Purity Evaluations

To eliminate any possibility of interferences affecting the validity of the LC results for B_1 and B_2, UV scans by diode array detection from 210 to 400 nm for the infant formula extract were compared with those obtained for the standard. The UV scans indicated no coeluting compounds, and the peaks were homogenous. The purity of the B_6 peak was confirmed by the procedure described by Haroon et al. (19) for fluorescence response. Fluorescence emission at 395 nm was determined at 2 excitation wavelengths, 275 and 305 nm, in addition to that at 295 nm. Calculated emission ratios with excitation at 295/275 and 295/305 for the sample were 3.16 and 2.01, respectively, and agreed very well with the respective ratios for the standard of 3.13 and 2.02, thus indicating the purity of B_6 peak.

During the course of this study, several other extraction techniques were examined. Heating the extract in any way, whether by autoclaving or water bath heating, resulted in poor chromatograms and interference peaks. The retention time of the B_1 peak was observed to change with peak broadening. Enzyme extractions were studied by using Takadiastase (Pfaltz and Bauer, Inc., Waterbury, CT. 06708); however, this digestion resulted in extreme interferences in the UV chromatograms so that no vitamin peaks could be observed.

As noted in the earlier study by Chase and Soliman (1), the use of the internal standard greatly facilitated the analysis, especially because the same internal standard could be used with B_6 as well as B_1 and B_2.

Conclusions

This multivitamin LC method provides a fast, accurate, and reliable means of simultaneously determining B_1, B_2, and pyridoxine in infant formulas and avoids the use of individual analytical AOAC methods, as well as the lengthy and time-consuming microbiological assays. This study further shows that perchloric acid digestion yields clear extracts, interference-free, baseline-resolved peaks, and complete recovery of added B_1, B_2, and B_6.

Values obtained by the LC method for B_1 and B_2 agreed closely with results of accepted AOAC methods. The method is limited to analysis of pyridoxine and will not provide a complete measure of naturally occurring vitamin B_6 activity in fortified products.

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VITAMINS

Reversed-Phase Liquid Chromatographic Determination of Vitamin D in Infant Formulas and Enteral Nutritionals

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Vitamin D in infant formulas and enteral nutritional products is determined by reversed-phase liquid chromatography (LC) with UV detection. The sample is saponified 30 min at 60°C and extracted into 60 mL hexane. The hexane layer is then washed and evaporated to dryness. The sample is reconstituted and added to a 3 mL silica solid-phase extraction column. Vitamins D_2 and D_3 are eluted from the column with 7 mL methylene chloride–isopropanol mixture (99.8 + 0.2). The eluant is evaporated to dryness and reconstituted in 1 mL acetonitrile. The acetonitrile solution is analyzed on a C18 reversed-phase LC column (25 cm × 4.6 mm, 5 μm particle size) with UV detection at 265 nm. Linearity for this method between 8 and 2600 IU/qt has shown a coefficient of determination of 1.000, with method precision ranging from 1 to 6%. Spike recoveries gave a mean of 99.1%. Because this method can quantitate and distinguish between vitamin D_2 and vitamin D_3 in products, vitamin D_2 is used as an internal standard in quantitating vitamin D_3, and vice versa. The method is applicable to milk-, soy-, and protein hydrolysate-based infant formulas and enteral nutritional products, both liquid and powder. The sample throughput is estimated to be 24 per day. An AOAC collaborative study of this method is recommended.

Vitamin D is the name given to a series of compounds that have antirachitic activity. The metabolites of vitamin D are thought to be the active species and play important roles in the homeostasis of calcium and phosphorus. Vitamin D_2 and vitamin D_3 are the 2 vitamin D compounds most commonly used to fortify food products at about the 10 ppb level. Vitamin D_3 is formed by the action of sunlight on the skin and is, therefore, more correctly termed a prohormone instead of a vitamin (1).

Significant technical difficulties in the determination of vitamins D_2 and D_3 result from the low level of fortification in complex matrixes and their lability to heat, light, and oxidation. Methods for the determination of vitamins D_2 and D_3 can be categorized as either biological assays or analytical chemistry assays. Biological assays (1) include the rat bioassay, the chick bioassay, intestinal calcium absorption, bone calcium mobilization, chick growth rate, and immunoassay. The official AOAC method for determination of vitamin D in infant formulas is the rat bioassay (2). Although these methods are very specific, they are time-consuming and relatively imprecise compared with analytical chemistry assays. Analytical chemistry assays (1) include UV absorption, colorimetry, fluorescence, gas chromatography, liquid chromatography (LC), and competitive binding. The determination of vitamin D in food products has relied mainly on the use of LC (3–11).

The method currently recommended for AOAC official first action status for the determination of vitamin D in infant formulas is a normal-phase LC method (10). It involves saponification, extraction, amino–cyano LC cleanup, fraction collection, concentration, and normal-phase LC quantitation. The estimated sample throughput is 4–8 samples per day with a precision of 7.7% and a recovery range of 93–98%.

The method described in this paper is accurate and precise for the analysis of infant formulas and enteral products, regardless of their protein source. It involves saponification, extraction, solid-phase extraction cleanup, concentration, and reversed-phase quantitation. The estimated sample throughput is 24 samples per day with overall precision of 6% and recovery of 99.1%.

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METHOD

Apparatus

(a) *Liquid chromatograph.*—Hewlett-Packard Model 1090 or 1050, equipped with UV detector and autosampler, and connected to Model 3357 automation system (LAS). Column should be operated at room temperature; higher temperatures result in loss of resolution. Equivalent system may be used.

(b) *LC column.*—Vydac 201TP54, 5 μm particle, 25.0 cm × 4.6 mm id C18. Column system providing equivalent retention characteristics may be used. (Note that this column is not endcapped. We have found this to be essential for separation.)

(c) *Solid-phase extraction column.*—Burdick & Jackson silica column 9054, 500 mg/2.8 mL, or equivalent. (This vendor's SPE column has been found to give very reproducible elution rates and analytical results.)

(d) *Vacuum manifold.*—For solid-phase extraction columns.

(e) *Evaporation apparatus.*—Zymark Turbovap, nitrogen flow, evaporator, or equivalent.

(f) *Rotary vaporator.*—Buchi, or equivalent.

(g) *Water bath shaker.*—Capable of maintaining temperature at 60°C.

Reagents and Solutions

(a) *Solvents.*—*n*-Hexane, methylene chloride, acetonitrile, isopropyl alcohol, methanol, ethyl acetate; all LC grade (Fisher Scientific, or equivalent).

(b) *Ethanol, anhydrous.*—USP grade (US Industrial Chemicals, or equivalent).

(c) *Potassium hydroxide.*—Pellets, AR grade (Mallinckrodt, Inc., or equivalent).

(d) *Glacial acetic acid.*—AR grade (Mallinckrodt, Inc., or equivalent).

(e) *Phenolphthalein solution.*—Dissolve 1 g phenolphthalein in 100 mL anhydrous ethanol.

(f) *Methylene chloride–isopropyl alcohol (IPA) solution.*—(99.8 + 0.2, v/v). Pipet 2 mL isopropyl alcohol into 1 L volumetric flask. Add methylene chloride to volume and mix.

(g) *Acetic acid solution.*—10%. Add 10 mL glacial acetic acid to 100 mL volumetric flask, dilute to volume with distilled water, and mix well.

(h) *Ethanolic potassium hydroxide solution.*—Dissolve 140 g potassium hydroxide pellets in 310 mL anhydrous ethanol and 50 mL water. Prepare fresh daily.

(i) *Mobile phase.*—Gradient combination of acetonitrile, methanol, and ethyl acetate. Pump table defined under *Chromatographic Determination.*

(j) *Vitamin D_2 and vitamin D_3 standards.*—USP reference standard, or equivalent.

(k) *Vitamin D_2 stock solution I.*—Accurately weigh 40–50 mg USP Vitamin D_2 reference standard. Transfer quantitatively to 250 mL low-actinic volumetric flask, and dilute to volume with anhydrous ethanol. Prepare fresh every 4 weeks and store in freezer.

(l) *Vitamin D_2 stock solution II.*—Pipet 4.0 mL vitamin D_2 stock solution I into 250 mL low-actinic volumetric flask, and dilute to volume with anhydrous ethanol. Prepare fresh each week and store in refrigerator.

(m) *Vitamin D_2 stock solution III.*—Pipet 4.0 mL vitamin D_2 stock solution II into 250 mL low-actinic volumetric flask, and dilute to volume with anhydrous ethanol. Prepare fresh weekly and store in refrigerator.

(n) *Vitamin D_3 stock solution I.*—Prepare and store as for vitamin D_2 stock solution I by using vitamin D_3 reference standard.

(o) *Vitamin D_3 stock solution II.*—Prepare and store as for vitamin D_2 stock solution II by using vitamin D_3 stock solution I.

(p) *Vitamin D_3 stock solution III.*—Prepare and store as for vitamin D_2 stock solution III by using vitamin D_3 stock solution II.

(q) *Internal standard.*—In quantitating vitamin D_2, use vitamin D_3 stock solution III as internal standard. In quantitating vitamin D_3, use vitamin D_2 stock solution III as internal standard.

Preparation of Samples and Standards

All procedures should be carried out in subdued lighting to minimize vitamin degradation. Both standards and samples are carried through entire procedure.

(a) *Standards.*—Pipet 4.0 mL internal standard and 4.0 mL vitamin D stock solution III into 125 Erlenmeyer flask, and add 15.0 mL H_2O.

(b) *Samples.*—Pipet 4.0 mL internal standard into 125 mL Erlenmeyer flask and add from syringe 15.0 mL sample prepared to quart normal dilution (containing ca 7–8 IU vitamin D).

Saponification and Extraction

Add 15.0 mL ethanolic potassium hydroxide solution to each sample and standard. Stopper flasks and place 30 min in 60°C shaker bath. Remove, and cool to room temperature. Transfer to 250 mL separatory funnel. Add 15.0 mL water to Erlenmeyer flask, stopper, and shake vigorously. Transfer to separatory funnel, rinse flask with 60 mL hexane, and transfer to funnel. Stopper funnel and shake vigorously 90 s. Let layers separate ca 10 min. Drain and discard aqueous layer, add 15.0 mL water to hexane layer remaining in funnel, stopper, and shake vigorously. Let layers separate, and drain and discard aqueous layer.

Add 1 drop phenolphthalein solution and 15.0 mL water to separatory funnel. Add 10% acetic acid solution to funnel dropwise with shaking until washing is neutral. Drain and discard aqueous layer. Drain hexane layer through sodium sulfate supported by small cotton plug into 100 mL round-bottom flask. Rinse funnel and sodium sulfate with few milliliters of hexane, collecting hexane in round-bottom flask.

Evaporation and Solid-Phase Extraction

Evaporate to dryness at 40°C on rotary evaporator. Immediately add 2.0 mL methylene chloride–isopropyl alcohol solution (99.8 + 0.2).

Prepare SPE columns by washing with 4.0 mL methylene chloride–IPA (80 + 20), and then 5.0 mL methylene chloride–IPA (99.8 + 0.2). Transfer solution in round-bottom flask to column by using disposable dropper. Rinse round-bottom flask again with 1.0 mL methylene chloride–IPA (99.8 + 0.2), and transfer rinse to column. Wash column with 2.0 mL methylene chloride–IPA (99.9 + 0.2). Discard this fraction. Elute vitamins D_2 and D_3 with 7.0 mL methylene chloride–IPA (99.8 + 0.2) into 16 × 100 mm disposable culture tube.

Evaporate methylene chloride–IPA by using nitrogen and warm water bath (Turbovap) at 40°C. When dry, add 1.0 mL acetonitrile to tube and swirl to rinse down sides of tube. Transfer to LC sample vial by using disposable dropper.

Chromatographic Determination

Inject standards at beginning, middle, and end of each run (24 samples). The following operating parameters are typical:

injection volume, 250 μL; wavelength, 265 nm; column temperature, 27°C; mobile phase, (A) acetonitrile, (B) methanol, and (C) ethyl acetate; flow rates as follows:

Pump Table

Time, min	Flow, mL/min	A, %	B, %	C, %
0.0	0.7	91.0	9.0	0.0
28.0	0.7	91.0	9.0	0.0
28.5	2.5	0.0	0.0	100.0
31.0	2.5	0.0	0.0	100.0
31.5	2.5	91.0	9.0	0.0
33.0	2.5	91.0	9.0	0.0
34.0	0.7	91.0	9.0	0.0

stop time, 35 min; retention times for vitamin D_2, 19.5 min, and vitamin D_3, 23 min.

Use internal standard methodology and peak heights for calculations.

System Suitability Tests

Standard solution carried through saponification, extraction, evaporation, and SPE is used for system suitability during routine operation. Resolution factor (as defined by USP) between vitamin D_2 and vitamin D_3 should be >2.0. Separation between these peaks should be sufficient to allow additional peak to be resolved between vitamin D_2 and D_3, namely, previtamin D_3. To verify that previtamin D_3 is resolved, system suitability standard is prepared by using USP System Suitability standard preparation on solution of USP Cholecalciferol Reference Standard combined with USP Ergocalciferol Reference Standard. This System Suitability Reference Standard contains, in order of elution, pre-D_2, D_2, pre-D_3, and D_3 (Figure 1).

LC can best be optimized by adjusting the amount of methanol in mobile phase; decreasing methanol content increases retention times. Column temperature should be maintained at 27°C; increased temperatures decrease retention, and vice versa. Six replicate injections of standard should have <2% relative standard deviation (RSD), and RSD of standards throughout run should be <4%.

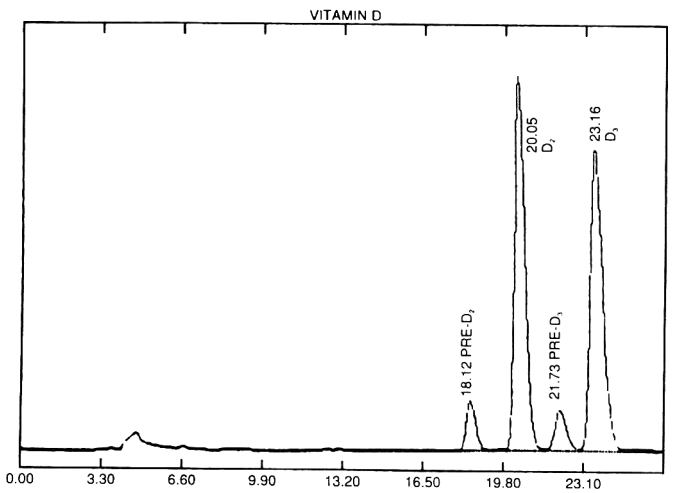

Figure 1. Stressed USP standards; LC conditions defined in text.

Results and Discussion

Figure 2 is a chromatogram of a vitamin D_3 standard and vitamin D_2 internal standard that was taken through the sample preparation process. Figure 3 is a chromatogram of a milk-based infant formula placebo. This placebo is a routine product made specifically without vitamin D_3 fortification. The placebo was spiked with internal standard (vitamin D_2) as instructed in *Preparation of Samples and Standards*. Figures 4 and 5 are chromatograms of a milk-based infant formula and enteral product, respectively, from routine production.

Vitamin D_2 is used as the internal standard for quantitating the levels of vitamin D_3 added in our products. Chromatographic parameters used in obtaining each of these chromatograms are identical to those outlined earlier.

Quantitation requirements for this nutrient cover a broad range. Levels of vitamin D in infant formulas and enteral products vary dramatically. Detection and quantitation limits, as well as the linearity range, therefore, had to be similarly broad. We set our detection and quantitation limits at the lowest concentration measured in determining linearity, 1.3 IU/mL [equivalent to ~60 IU/quart normal dilution (QND) in this method]. The maximum concentration measured for linearity was 43 IU/mL (2600 IU/QND). These limits can be expanded by using a detector with greater sensitivity or testing a broader range for linearity.

Method accuracy was measured by determining the recoveries of spikes added to product placebos (products made in a pilot plant setting with all nutrients except vitamin D added). Placebos were used in place of the routine product to reduce production-based variability and eliminate loss due to thermal degradation, thereby assuring accurate theoretical values. These placebos were spiked at levels equal to the expected range of results, both high and low, and to the theoretical values. Average recoveries were 98% for milk-based, 101% for soy-based, and 102% for protein hydrolysate-based infant formula products. Recoveries averaged 99% for milk-based and protein hydrolysate-based enteral products (Table 1).

Vitamin D methods previously submitted for collaborative study yielded average recoveries of 90 and 93–98% (8, 11).

Linearity of the detector response was evaluated by running a 7-point standard curve. Concentrations measured for linearity ranged from 20 to 650% of the normal infant formula sample concentrations. Excellent linearity was demonstrated for vitamin D_2 and vitamin D_3 individually. The coefficients of determination were 0.9999 for vitamin D_2 and 0.9999 for vitamin D_3, and 1.000 for "R" values. The y-intercept for "R" values was <.01% of the standard concentration equal to normal infant formula samples. A single concentration of reference standard, therefore, can be used for routine analysis.

Method precision, both short-term and long-term, was measured on each product type. Short-term precision was evaluated by running 6 replicates each of placebos spiked at the expected

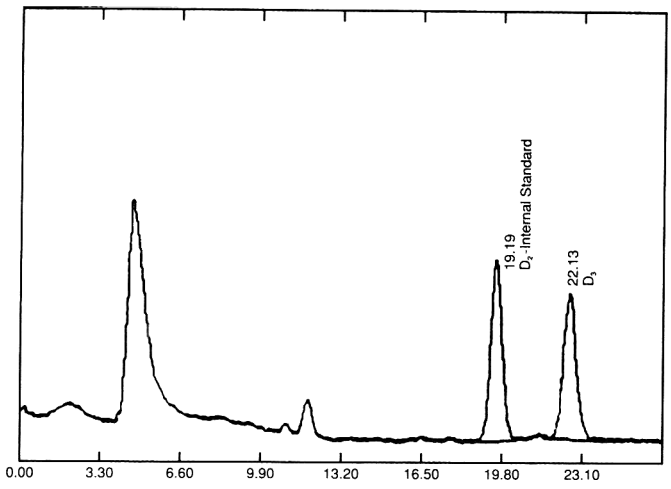

Figure 2. Standard chromatogram; LC conditions defined in text.

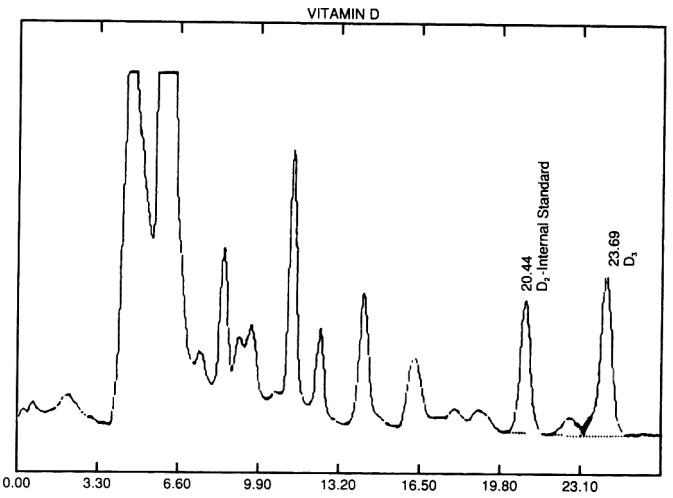

Figure 4. Milk-based infant formula; LC conditions defined in text.

product concentration in 1 day. Excellent short-term precision was shown by RSDs of 1.0–3.9%. Long-term precision was determined by assaying the same batch of routine product on 6 separate days. Values of 3.8–5.9% RSD were obtained, showing very good long-term precision. Results for individual product types are shown in Table 2.

Instrument precision was assessed in 2 ways. The first measurement was made by injecting a standard solution at the optimum concentration for routine analysis 10 times. Precision of height values for vitamins D_2 and D_3 as well as the "R" values in this correlation coefficient were calculated; values for RSD were 1.69, 2.13, and 0.96%, respectively (Table 3). The second evaluation was made by reviewing reproducibility of the 3 standard injections made on every run.

Data covering 5 months and including 6 standard solutions showed RSDs generally below 1.5% (Table 4). These data show that the method has excellent system precision.

Method ruggedness was challenged across instruments, columns, and analysts. Multiple lots of SPE columns from the vendor listed under *Apparatus* were used in generating the data in Table 5, detailing the SPE column's ruggedness. Results obtained with separate instruments and analytical columns showed little difference; RSDs of <2% were obtained in measuring normal levels in the product. Analyst-to-analyst variation was also very small; results from 3 different batches of product showed ≤2% RSD. Specific data in Table 5 show this method to be extremely rugged.

Quantitation of previtamin D was theoretically possible because LC separates the previtamin D peaks from their respective vitamin Ds in addition to separating the vitamin D peaks. The levels of previtamin D in product, based on theoretical calculations (12), are below our detection limits and, therefore, not possible to quantitate. Previtamin D is biologically active, however, and must be included when total vitamin D activity is reported. A factor of 1.05 times the amount of vitamin D is used to calculate total vitamin D. This factor is based on literature values (13), information from vitamin D manufacturers, and historical in-house data.

The purity and identity of the vitamin D peaks were verified by comparing retention times, absorbance ratios (265 vs

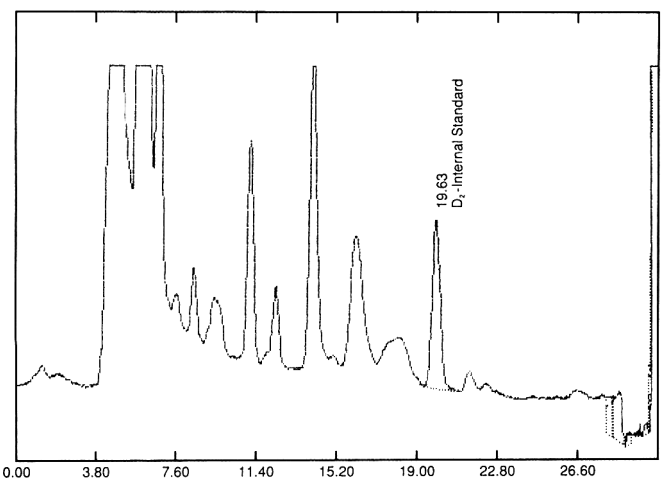

Figure 3. Milk-based infant formula placebo; LC conditions defined in text.

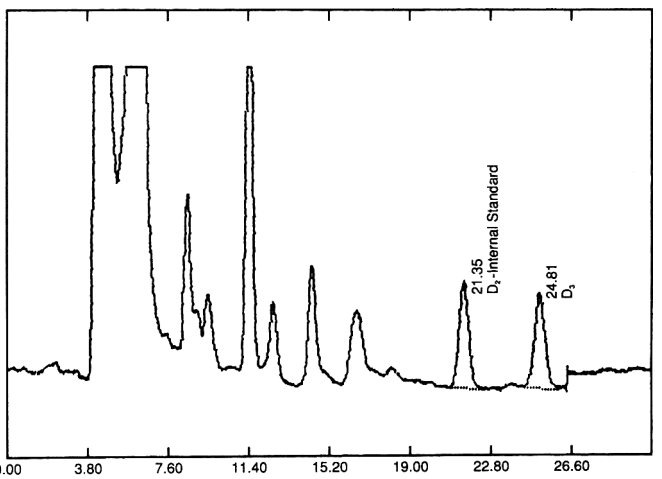

Figure 5. Milk-based enteral; LC conditions defined in text.

Table 1. Spike recoveries (IU/qt)

Product	Added	Recovered	Rec., %
Soy-based infant formula (n = 4)	115	118	103
	461	458	99
	922	922	100
			$\bar{X}$ = 101
Milk-based infant formula (n = 4)	115	109	95
	461	455	99
	922	927	101
			$\bar{X}$ = 98
Protein hydrolysate-based infant formula (n = 2)	115	125	109
	461	456	99
	922	903	98
			$\bar{X}$ = 102
Milk-based enteral product (n = 8)	230	230	100
	461	452	98
	922	919	100
			$\bar{X}$ = 99
Protein hydrolysate-based enteral product (n = 3)	115	116	101
	346	343	99
	922	905	98
			$\bar{X}$ = 99

254 nm, 280 vs 265 nm, 280 vs 254 nm), and spectral scans of vitamin D standards with a variety of samples. Good agreement was achieved between standards and samples for all 3 criteria. Retention times were within 0.1 min, absorbance ratios agreed to within 3%, and spectral scans of samples and standard overlapped well.

Samples of commercially available infant formulas and enteral products from different manufacturers were obtained. Chromatographic procedures were similar to that used with our products in all cases. Vitamin D in infant formulas was above label claim by a consistent amount when determined with our method. Vitamin D in enteral products also was above label claim.

In addition to assaying infant formula and enteral product, 2 other commercially available vitamin-D fortified liquids were analyzed. Whole milk and a weight-control liquid were analyzed, with results comparable to label claim. Chromatography was excellent.

Data gathered to date on nutritional powders were not included here. All indications are that implementing this method for powders will cause no problem. Slurries prepared from both infant formula and enteral product powder have been assayed.

Table 2. Method precision (RSD, %) at theoretical levels of vitamin D

Product type	Short term	Long term
Milk-based infant formula	1.1–1.3	4.4
Soy-based infant formula	1.0–2.1	5.9
Protein hydrolysate-based infant formula	1.1–2.8	5.0
Milk-based enteral products	1.0–3.9	5.7
Protein hydrolysate-based enteral product	1.6–3.6	3.8

Table 3. Instrument precision 1 (response as 0.25 μV)

Injection	Height D_2	Height D_3	R value (D_3/D_2)
1	7243	5953	0.8218
2	7122	5871	0.8243
3	7245	5883	0.8121
4	7032	5835	0.8298
5	7023	5829	0.8299
6	7171	5954	0.8302
7	7112	5865	0.8246
8	7207	6029	0.8366
9	7378	6159	0.8348
10	7355	6173	0.8393
Mean	7188.8	5955.1	0.82834
SD	121.381	127.033	7.99251×10^{-3}
RSD, %	1.68847	2.13318	0.964883

Table 4. Instrument precision 2

Determination	Day	Std solution	RSD, %
1	1	A	1.24
2	2	A	0.87
3	7	B	1.23
4	12	B	0.76
5	14	B	0.82
6	16	B	0.73
7	20	B	2.55
8	21	B	2.57
9	22	B	1.38
10	32	B	0.35
11	35	B	1.00
12	36	C	1.03
13	43	C	1.13
14	45	C	1.58
15	84	D	0.48
16	90	D	0.89
17	92	D	1.85
18	95	D	2.15
19	98	E	0.79
20	105	E	1.81
21	108	E	0.93
22	110	E	1.00
23	115	E	0.09
24	127	F	0.67

Table 5. Ruggedness

	Instrument/column (IU/qt)		
Determination	1	2	3
1	500	136	452
2	520	148	468
3	508	136	456
Mean	509.3	140.0	458.7
SD	10.1	6.93	8.33
RSD, %	1.98	4.95	1.82
	Analyst		
Determination	1	2	3
1	512	136	468
2	524	140	464
3	500	136	452
Mean	512	137.3	461.3
SD	12.0	2.31	8.33
RSD, %	2.34	1.68	1.80

Results compare very favorably to theory. Chromatography of these products also is excellent.

Conclusion

The method described here offers a significant improvement over currently available methods. Use of an internal standard significantly increases accuracy and precision, as analyst technique in extracting vitamin D from the sample matrix is no longer as critical. The use of solid-phase extraction to perform sample cleanup has the potential to increase sample throughput anywhere from 50 to 400%, depending on the current method used. The LC system separates vitamin D_2 and vitamin D_3, as well as their respective previtamin D components, improving specificity as well as accuracy. Because this method provides significant advantages over current methodology, we recommend that a collaborative study be started as soon as possible.

Acknowledgments

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TECHNICAL COMMUNICATIONS

Detection of Beef, Sheep, Deer, and Horse Meat in Cooked Meat Products by Enzyme-Linked Immunosorbent Assay

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Enzyme-linked immunosorbent assays (ELISAs) are described for the detection of mutton, beef, horse meat, and venison in cooked meat products. They represent an expansion of the species detection capabilities of previously described ELISAs for the detection of pork and poultry in cooked foods. These double antibody sandwich ELISAs recognize heat-resistant antigens in simple aqueous extracts of cooked meat products. Tests on laboratory-prepared and commercially cooked meat products accurately differentiated all tested meat components. However, some canned baby food meats and one canned meat product did not react in any of these ELISAs. Sensitivity of the assays was 0.13% or greater in tests of diluted cooked extract mixtures. No product ingredients were found that interfered with test performance.

Legislative authority mandates that meat and poultry products be accurately labeled as to species content. Correct species identification is important to the consumer for several reasons: (*1*) possible economic loss due to fraudulent substitution or adulteration; (*2*) medical requirements of individuals who may have specific food allergies; and (*3*) religious dietary restrictions. Species of raw meats can be identified by several techniques (1–4), but methods for cooked meat speciation have met with limited success (5–7). The use of enzyme-linked immunosorbent assays (ELISAs) in food analysis is finding increasing application; the advantages of the system include simple sample preparation, relatively inexpensive instrumentation, and adaptability to either field or laboratory settings (8, 9). When antibodies are properly prepared and standardized, and test criteria are stringently applied, ELISA can be used accurately and reliably in conjunction with quality control and quality assurance programs (10).

To improve capabilities for accurate species determination, ELISAs have been developed that detect mutton (lamb), beef, horse meat, and venison in cooked meat products. They are important additions to those previously described for the detection of cooked pork and poultry (11). These ELISAs, which are based on species-specific polyclonal antibodies produced in rabbits by immunization with heat-resistant antigens, are an extension of the work described by Berger et al. (11) for detecting pork and poultry in cooked meat products. They use a double antibody sandwich technique with an unlabeled antispecies antibody coated on a microtiter plate. After incubation with the sample to be analyzed, a second antibody labeled with biotin is added. The reaction is amplified by a streptavidin horseradish peroxidase conjugate and developed with 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS).

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Experimental

Antibody Production

Antigens and antisera were prepared as described by Berger et al. (11). Briefly, soluble antigens were partly purified from saline extracts of raw, ground whole muscle tissue by fractional ammonium sulfate precipitation. The extract was dialyzed, concentrated, and passed through a carboxymethyl cellulose cation exchange column. Rabbits were immunized with the major peaks eluted, using a series of antigen injections and a combination of Freund's complete and incomplete adjuvants and RIBI adjuvant (RIBI Immunochemical Research, Inc., Hamilton, MT).

Immunoglobulin G (IgG) was harvested from the antisera with Zeta Prep ion exchange cartridges (CUNO, Inc., Life Sciences Division, Meriden, CT) and concentrated to 1 mg/mL. For each species, one part of the IgG was aliquoted, stored in 0.01M phosphate-buffered saline (PBS), pH 7.2, at −70°C, and used as coating antibody. The other part was dialyzed against 0.1M sodium bicarbonate, and aliquots were stored at −70°C. Each aliquot of the latter preparation was biotinylated as needed for use as second antibody. The biotinylated antibody was combined with glycerin (1 + 1) and stored at −20°C. If necessary, biotinylated antibody was adsorbed with the cross-reacting species immunosorbent before glycerin was added.

Immunosorbents were prepared from beef, sheep, and deer meat by the method of Ternyck and Avrameas (12) with slight modifications. Skeletal muscle tissue was homogenized in 0.2M acetate buffer, pH 5.0 (250 g/500 mL), and extracted 1 h at room temperature. The slurry was centrifuged 20 min

at 12 000 × *g*. The supernatant was filtered through Whatman No. 42 filter paper and concentrated to a paste by dialysis against 30% polyethylene glycol. This concentrate was suspended in 20–30 mL 0.2M acetate buffer and centrifuged 15 min at 27 000 × *g*. The supernatant was filtered through a 0.45 μm filter (Millipore Corp., Bedford, MA). Total protein was determined by the method of Lowry (13). The concentration was adjusted to 50 mg/mL by dilution in acetate buffer. Protein was fixed by the dropwise addition of 2.5% aqueous glutaraldehyde (0.2 mL for each mL of protein solution) with gentle stirring. The solution was incubated 3 h at room temperature without stirring and refrigerated overnight. The resulting gel was finely diced and forced through an 18-gauge needle several times. It was dispersed in 0.1M PBS, pH 7.2 (100 mL for each 12 mL of gel). The slurry was washed by centrifuging 10 min at 3000 × *g* and resuspending in fresh PBS. This was repeated 3 times. The suspension was centrifuged again, and the pellet was resuspended in 100 mL 0.2M glycine buffer, pH 2.8, and incubated 15 min at room temperature. The preparation was washed once in PBS, recentrifuged, and suspended in 50 mL 1M solution of ethanolamine in PBS. It was incubated overnight at 4°C, washed 3 more times in PBS, resuspended in 100 mL PBS with 0.02% sodium azide, and stored at 4°C.

For adsorption, 5 mL suspended immunosorbent was incubated overnight at 4°C with 0.5 mL normal rabbit serum. The suspension was then centrifuged, and the gel pellet was resuspended in 3 mL of the biotinylated species antibody to be adsorbed. This slurry was incubated 8–24 h at 4°C and then centrifuged to pellet the gel. The gel pellet was discarded and the treated supernate was tested for specificity. The process was repeated as needed up to 3 times. The treated antibody was filtered through a Millipore filter, glycerinated, and used in the described ELISA.

ELISA Performance

Each well of EIA II Plus microtiter plates (Flow Laboratories, Inc., Dublin, VA) was coated with 100 μL coating antibody in 0.05M Tris-HCl (1 + 500), pH 7.7, with 0.01% merthiolate. Plates were sealed with clear plastic tape and stored 24 h to 6 months at 4°C in a humidity chamber. Control extracts of meat species were prepared by adding 60 mL saline to 20 g diced meat of known identity in leak-proof plastic bags and dispersing the tissue with a Colworth stomacher for 10 s. The preparation was incubated 1 h at room temperature, placed in a boiling water bath for 15 min, cooled, and centrifuged 15 min at 10 000 × *g*. The supernatant was filtered through a 0.45 μm filter. Portions were stored at −20°C. Cooked meat samples to be analyzed were extracted in 10 mL distilled water with 5 g sample, dispersed 1 min with the stomacher, and extracted 1 h. The preparation was centrifuged at 15 000 × *g*, and the supernatant was used for the assay.

To perform the ELISA, a plate coated with the desired species antibody was removed from the humidity chamber. Wells were washed before samples were added and after each subsequent procedure. The wash cycle was completed by filling and aspirating each well 3 times with 300 μL PBS-Tween (0.075M sodium phosphates, 0.075M NaCl, and 0.05% Tween 80, pH 7.2). After the wash cycle, 100 μL of each sample to be tested was placed in each of 4 wells. Blank wells and positive and negative controls were included on each plate in a prescribed format. Samples were incubated 1 h on the plates at room temperature. The plate was then washed, 25 μL diluted biotinylated antibody was added to each well, and the plate was incubated 1 h. Next, 25 μL streptavidin horseradish peroxidase conjugate was added, and the plate was incubated 30 min. After the wash step, the wells were refilled with 300 μL each of PBS-Tween and allowed to soak 1 h. The wash fluid was aspirated, and 50 μL ABTS substrate with 0.015% hydrogen peroxide was added to each well. After 30 min, the reaction was stopped by adding 50 μL 0.1M citric acid/well. The absorbance was read at 414 nm (maximum chromogen absorbance) on a dual beam spectrophotometer; the absorbance at 492 nm (minimum chromogen absorbance) was subtracted. The means and standard deviations were calculated for the controls and samples.

Assay Evaluation

Each species assay was evaluated for specificity, sensitivity, and accuracy. Specificity was determined by testing meat extracts from animals of other taxonomic families. Available meat from other species within the same family was also assayed. Sensitivity was determined by testing 10-fold dilutions of homologous meat extracts diluted in extracts of 2 other species. Sheep, horse, and deer extracts were each diluted in beef and pork extracts. Beef extract was diluted in sheep and pork extracts. Accuracy was determined by blind assay of 40 coded samples: 37 samples of a variety of commercial meat products, 1 vegetarian meat substitute, and 2 laboratory-prepared meats. The commercial products included hot dogs, bolognas, canned sandwich spreads, pressed meats, frozen ready-to-eat meats, canned hams, canned soups, and baby foods.

Results and Discussion

Specificity test results are presented in Table 1. To eliminate nonspecific reactions, beef antibody was adsorbed by using both sheep and deer immunosorbents, and sheep antibody by using beef and deer immunosorbents. Because immunoadsorption also reduces the homologous antibody titer, antibody preparations were tested after each adsorption to determine whether additional adsorption was necessary.

All assays demonstrated approximately 10-fold or greater difference in absorbance between homologous and heterologous meat samples. Samples from species within the same subfamily gave results of varying intensities. American Bison reacted in the beef assay, goat meat in the sheep assay, and donkey and mule meat in the horse assay. The deer assay reactions included not only whitetail (source of the immunogen) and mule deer, which gave very similar reactions, but also reindeer and caribou. Reindeer and caribou are very closely related and produced indistinguishable results in the ELISA. Moose and elk meat produced much lower reactions than the reference extract.

Table 1. Results of ELISA specificity tests of cooked meat extracts (mean ± SD) (16 text wells)[a]

Species	ELISA			
	Beef	Sheep	Horse	Deer
Beef	0.639 ± 0.006[a]	0.048 ± 0.012	0.033 ± 0.009	0.054 ± 0.005
Bison	0.503 ± 0.010	0.088 ± 0.004	0.042 ± 0.004	0.072 ± 0.016
Sheep	0.048 ± 0.007	0.611 ± 0.004	0.035 ± 0.004	0.050 ± 0.019
Goat	0.035 ± 0.004	0.328 ± 0.018	0.004 ± 0.001	0.052 ± 0.003
Horse	0.025 ± 0.005	0.036 ± 0.002	0.632 ± 0.020	0.003 ± 0.001
Donkey	0.004 ± 0.002	0.032 ± 0.003	0.518 ± 0.024	0.003 ± 0.002
Mule	0.011 ± 0.006	0.042 ± 0.003	0.520 ± 0.027	0.011 ± 0.002
Whitetail deer	0.062 ± 0.006	0.090 ± 0.032	0.040 ± 0.030	0.676 ± 0.007
Moose	0.078 ± 0.009	0.098 ± 0.004	0.058 ± 0.008	0.160 ± 0.008
Elk	0.052 ± 0.008	0.112 ± 0.004	0.036 ± 0.007	0.182 ± 0.019
Pork	0.020 ± 0.011	0.029 ± 0.021	0.021 ± 0.011	0.002 ± 0.001
Chicken	0.006 ± 0.002	0.009 ± 0.004	0.005 ± 0.002	0.005 ± 0.001
Kangaroo	0.006 ± 0.002	0.006 ± 0.001	0.028 ± 0.002	0.002 ± 0.002
Dog	0.005 ± 0.003	0.029 ± 0.002	0.041 ± 0.004	0.004 ± 0.002
Rabbit	0.006 ± 0.002	0.029 ± 0.003	0.008 ± 0.002	0.002 ± 0.002
Milk	0.056 ± 0.006	0.040 ± 0.004	0.022 ± 0.008	0.037 ± 0.002
Egg white	0.006 ± 0.007	0.002 ± 0.001	0.004 ± 0.002	0.001 ± 0.001
Reindeer	ND[b]	ND	ND	0.398 ± 0.018
Mule deer	ND	ND	ND	0.514 ± 0.015

[a] OD 414 – OD 492 nm.
[b] ND = Not done.

The variable positive reactions demonstrated by some species within the same taxonomic families (Cervidae and Equidae) were not totally unexpected and should be kept in mind in interpreting results of analysis of unknown samples.

The least probable sensitivity for each assay was determined by diluting the homologous extract in the highest cross-reacting and the lowest cross-reacting red meat extracts. Results were calculated as the mean of 16 wells for each point and charted graphically (Figure 1). Estimates of sensitivity endpoints were determined by adding 3 standard deviations to the mean absorbance of each diluent control. The sensitivity endpoint was extrapolated from the extinction curve by placing this value on the ordinate and reading down from its intersection with the corresponding species curve to the concentration on the abscissa.

Results, summarized in Table 2, ranged from 1.3×10^{-3} (0.16%) for the detection of sheep extract diluted in beef extract to less than 1×10^{-4} for the detection of deer extract diluted in pork extract. This level of sensitivity is more than adequate for detecting fraudulent or mislabeled meat products; there is little economic incentive for adulteration at less than 1%.

The accuracy of the assays was demonstrated by the analysis of the 40 coded samples described in the experimental section. Each sample was tested on 5 separate occasions. An attempt was made to include a wide range of products; however, not all of the great variety of products on the market could be included. All positive results correctly identified the meat species present. However, 4 baby food meats and 1 canned sandwich spread failed to react in any assay. These products were also tested for pork and poultry content with similar negative results.

The negative results are probably due to extensive heat processing. The baby food meats contain no additives besides water, and all ingredients of the nonreactive sandwich spread were found in other products that were satisfactorily identified. Also, the antigen against which the antibodies are directed is known to be only relatively heat-stable. As described in the previous work (11), progressively severe heat treatment causes a progressive decrease in reactivity of the sample. As a result, highly processed meat products such as baby food and canned meats may produce lowered or "negative" readings in these ELISAs.

The determination of the species was apparently unaffected by the non-meat components. No instances were found in which an ingredient interfered with the assay or produced falsely elevated readings. The vegetarian product did not react in any assay. It should be noted, however, that the pH of pickled product extracts may need to be adjusted to 6.0 or greater before the extracts are tested.

The key to successful development of these ELISAs is the isolation of native heat-resistant immunizing antigens and the preparation of specific antisera from them. The immunizing antigens are heat-resistant as well as species-specific. However, neither the species specificity nor the heat resistance is absolute. Multiple boosters may be required for the production of antisera with the necessary high levels of IgG. This procedure also may increase the cross-reactivity of the antisera.

Treatment of the antibody preparation with the appropriate immunosorbent is quite effective in reducing the cross reac-

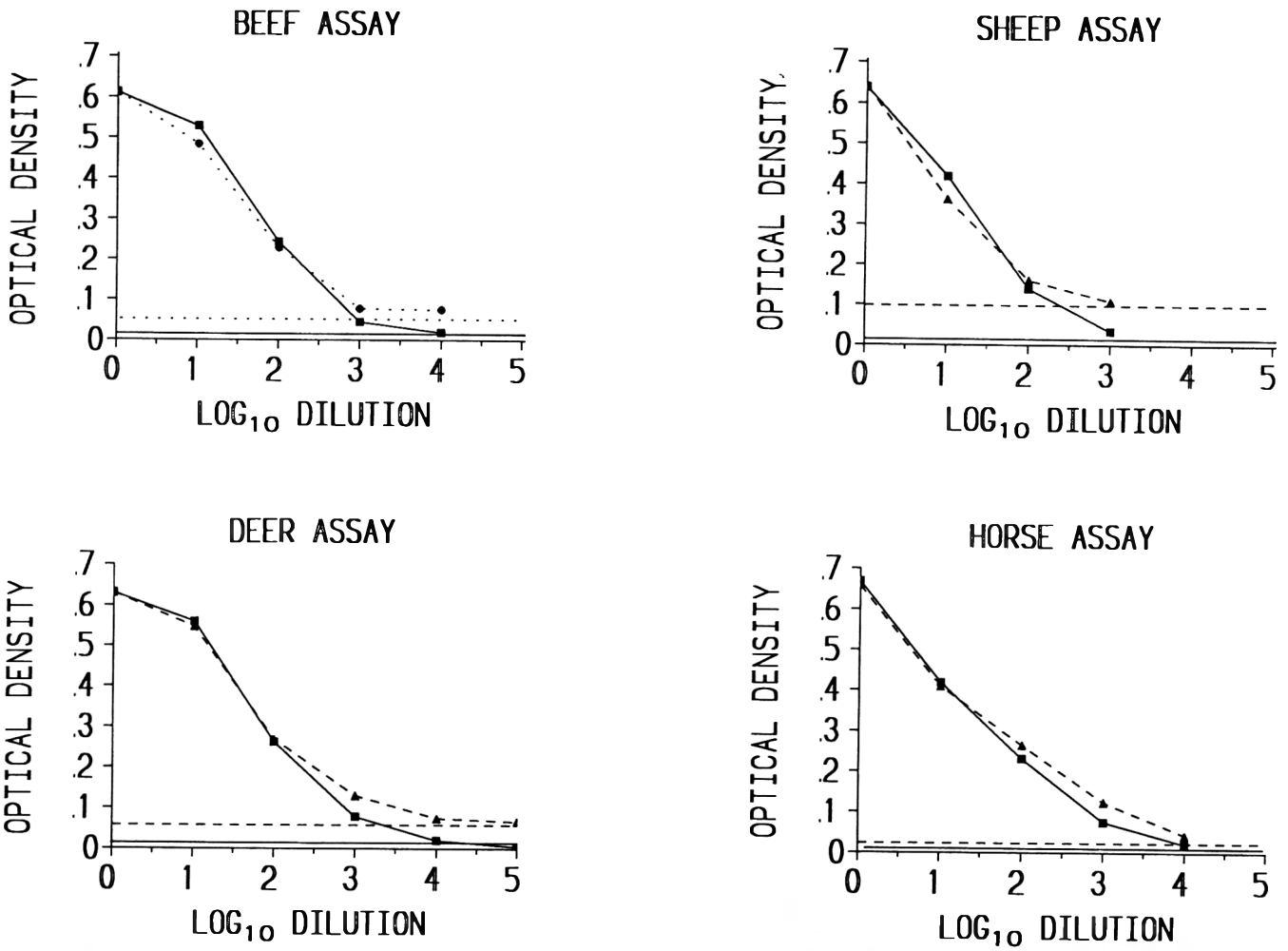

Figure 1. Results of ELISA sensitivity tests (mean of 16 wells). Baseline reference extract values: pork ——, beef – – –, sheep · · · · · · . Assay diluent: pork, —■—, beef —▲—, sheep · · ·●· · ·.

tions, but it also greatly reduces the homologous titer. Use of these assays with raw products may result in the development of nonspecific cross reactions. Therefore, the cooked meat ELISAs should be used *only* with cooked—not raw—meat products.

The ELISAs described here for the detection of beef, sheep, deer, and horse meats are intended to serve in a complementary manner to those previously described for poultry and pork. They have been shown to be fast, simple, accurate, and sensitive for the detection of these species in cooked meat products. The assays can be completed in 5 h and use a simple aqueous extraction of samples. The poultry and pork ELISAs have been used successfully by the Food Safety Inspection Service of the U.S. Department of Agriculture and by commercial laboratories since 1987. The addition of ELISAs for the detection of beef, sheep, horse, and deer meats provides a more complete spectrum of meat species identification in cooked meat products.

Table 2. Estimates of ELISA assay sensitivity endpoints derived from Figure 1

Species	Species diluent			
	Pork		Beef/sheep	
Sheep	1.3×10^{-3}	(0.13%)	1.6×10^{-3}	(0.16%)
Beef	1.1×10^{-3}	(0.11%)	1.0×10^{-3}	(0.10%)
Horse	5.0×10^{-4}	(0.05%)	1.4×10^{-4}	(0.014%)
Deer	$<1.0 \times 10^{-4}$	(<0.01%)	1.0×10^{-4}	(0.01%)

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TECHNICAL COMMUNICATIONS

An FDA Laboratory Approach to Uncovering Potential Fraud in the Generic Drug Industry

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The Division of Drug Analysis of the U.S. Food and Drug Administration in St. Louis, MO, has screened more than 1400 drug samples for potential generic drug fraud by a combination of physical, instrumental, and chemical techniques. The approach to fraud centered around the analysis of excipients as opposed to the normal approach of analysis of active ingredients. Approximately 80% of the drug formulation pairs (innovator and generic) submitted for bioequivalence testing could be differentiated by a combination of physical and instrumental analysis, mainly thermogravimetric analysis (TGA) and Fourier-transform infrared (FTIR) spectrometry. TGA proved to be the single most useful instrumental technique to determine differences in formulations. Liquid chromatography, gas chromatography, chemical tests, and polarizing microscopy were found to be the most useful tools to differentiate formulations that could not be resolved by FTIR, TGA, or physical comparisons. X-ray powder diffraction and nuclear magnetic resonance spectroscopy were found not to be useful techniques to differentiate formulations. No clear-cut evidence of direct fraud was found, but inconsistencies and suspicious samples were noted. Follow-up inspections based on the laboratory findings will probably occur. The procedures discussed in this article could be streamlined and made more efficient for examining large numbers of potential problem formulations.

In 1989, investigators of the U.S. Food and Drug Administration (FDA) uncovered fraudulent activity in the generic drug industry. As a result, FDA's Division of Drug Analysis (DDA) was asked to investigate the problem. Thomas Layloff has given an excellent overview and discussion about FDA's approach to this type of regulatory activity (1).

This article presents DDA's approach in detecting possible fraud in specific samples collected concerning the generic drug industry. An outline of the procedures followed is given, not the specifics of the methods. DDA received 1400 samples from various sources in about a month. Most samples were received from 2 pharmaceutical testing laboratories that had compared the bioequivalence of submitted generic formulations to innovator formulations. Although many types of fraudulent activity are possible, DDA decided to concentrate on being able to detect and prove the following: (*1*) direct substitution of one product (innovator) for another (generic), (*2*) altered innovator dosage formulations, and (*3*) unapproved changes in batch formulas.

The concept of analysis of samples for fraud was completely new to our laboratory. We quickly realized that an in-depth analysis of each sample for active and inactive ingredients (excipients) and conclusions to be made about fraud could not be accomplished in a timely manner. Many months or years might be involved.

In an attempt to solve this dilemma, we examined some possible fraud scenarios. If a generic company substituted an innovator's product for their own, then any physical data and any instrumental data would be identical, albeit possibly disguised. If a generic company had tampered or slightly modified an innovator's product, the data comparisons would be similar. If the generic company's and the innovator's products were truly different, then the comparisons could be different. If the generic company's and the innovator's products were truly different but contained the same excipients in about the same proportion or differed by a minor excipient, the data could be similar.

We were dealing with "similar" (sameness) or "different," and a gray area in between. Management selected an 80% or better match of the physical and instrumental data as a decision making marker. Two categories were established: (*a*) significantly different, no further work required, and (*b*) similar, needs further work. The 80% guideline was adopted to decrease the possibility of missing a fraudulent generic-innovator sample pair.

A laboratory protocol was set up for sample screening and comparison (data evaluation). The tools selected were physical measurements (size, weight, color, etc.) and instrumental techniques, including Fourier-transform infrared (FTIR) and thermogravimetric analysis (TGA). Nuclear magnetic resonance spectroscopy (NMR) and X-ray powder diffraction (XRPD)

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were used on a limited basis. Approximately 80% of the generic-innovator sample pairs were eliminated after the first screening. The remaining 20% required further testing.

Batch records were requested from FDA's Division of Generic Drugs for the samples that required further testing. Additional tools were used in the follow-up analysis, including liquid chromatography (LC), gas chromatography (GC), IC (ion chromatography), polarizing microscope, and chemical tests.

Experimental

Apparatus

(a) *Camera.*—Model CU-5 with 2× close-up attachment (Polaroid Corp., 575 Technology Square, Cambridge, MA 02139).

(b) *Color guide.*—PANTONE by Letraset Color Products Selector.

(c) *FTIR spectrometers.*—Model 1600 (Perkin-Elmer Corp., 761 Main Ave, Norwalk, CT 06859) and Nicolet Model 710 (Nicolet, 5225-1 Verona Rd, PO Box 4508, Madison, WI 53791-9598).

(d) *Gas chromatograph.*—Model 5890 (Hewlett-Packard Co., Route 41, PO Box 900, Avondale, PA 19311-0900).

(e) *Ion chromatograph.*—Model 4000i (Dionex, 1228 Titan Way, PO Box 3603, Sunnyvale, CA 94088-3603).

(f) *Polarizing microscope.*—Model GFL (Carl Zeiss, Inc., 1 Zeiss Dr, Thornwood, NY 10594).

(g) *Microscope.*—Wide field, low power (10–25×) (Bausch and Lomb).

(h) *Thermal analyzers.*—Model 9900 computer thermal analyzer, and Analyst 2000 with Model 951 thermal gravimetric analyzer (Dupont Co. Instrument Systems, Concord Plaza, Quillen Building, Wilmington, DE 19898).

(i) *X-ray powder-diffraction spectrometer.*—Datamax A (Rigaku USA, Inc., Damen Building, Suite 305, 200 W. Higgins Rd, Schaumbury, IL 60195).

(j) *Liquid chromatograph.*—Model 6000 solvent delivery system, Model 450 variable wavelength detector, Model 710B autoinjector, and Model 730 data module (Waters Chromatography Div., Millipore Corp., Milford, MA 01757).

Laboratory Format

Before laboratory analysis began, DDA set up a system for handling the samples submitted under investigators' seals. The investigators placed many samples from the testing facility in several sample cartons under one FDA seal and collection report. These samples were repackaged and individually resealed by DDA personnel.

Each generic company's bioequivalence sample was mated with the corresponding innovator's marketed product that was submitted by the generic company for comparison with the generic sample for bioequivalence study. An 8 digit numbering system was set up to mate the samples; we assigned odd numbers to indicate generic company samples and even numbers to indicate innovator company samples. These 8-digit numbers were directly related to the serial number of the investigator's collection report. The samples were then logged into our computer system. Sample cards and labels were printed for each sample.

To keep track of the information about the samples and their path through the laboratory, a 16-field database was set up on the computer system. Entries in the database consisted of drug name, sample number, serial number of the investigator's collection report, subnumber, innovator's name, study number (associated with the origin of the sample), status, generic company, sample type (generic or innovator), dosage, dosage units, dosage form, lot number, date received, date completed, and generic code for the drug name.

To prevent bias and to protect against sample mix-up, analysts were allowed to have only one sample at a time. As another safeguard, we set up a new sample storeroom that was only accessible to the sample custodian and 2 laboratory supervisors.

Data Collection

When an analyst received a sample for initial testing, a sticker label was forwarded to the team leader, who logged the sample into the database with drug name, generic company, and status. The status code used for *in process* samples was a dash (–).

The analyst began the testing using a laboratory protocol especially designed for this work. A physical examination was first. The units (tablets or capsules) were observed under a low-power (10–25×) Bausch and Lomb wide-field microscope for signs of tampering, such as innovator markings obliterated or painted over. Next, a unit was photographed with a Polaroid Model CU-5 camera with a 2× close-up attachment. The unit was measured and the net weight of the unit recorded. The unit was then numerically coded using an identification guide (2). The unit was compared to a commercial printer's color guide and the color number recorded. This was more accurate than allowing the chemist to make a judgment about the color, i.e., red, peach, pink, orange, etc.

A portion of a finely powdered unit was then mixed with KBr to make a 2% w/w pellet, whose FTIR spectrum was recorded from 4000 to 400 cm^{-1} by either of the FTIR spectrometers. A thermogram was obtained from another portion of the finely powdered unit by either of the thermal analyzers. The thermogram was taken from 25 to 600°C at 10°C/min. This rate was used instead of the optimum 2°C/min because of the pressure of the work load. The individual analysts ran the innovator and generic samples on the same instrument. XRPD spectrometry was used to supplement the FTIR and TGA instrumental data on certain samples.

Results and Discussion

Originally, all the samples were analyzed on the thermal analyzer, but the system quickly became backlogged at the TGA instruments because of their longer running time. The protocol was modified briefly to obtain only the physical characteristics and FTIR spectra. However, 80% of the sample pairs (innova-

Table 1. Percent of sample pairs found significantly different by techniques used in first screen

Technique	Percent
TGA	58
FTIR	18
Physical examination	3
X-ray	1
Undifferentiated by above techniques	20

Table 2. Percent of samples found different by additional techniques[a]

Technique	Percent
LC	35
Polarizing microscope	25
Chemical tests	20
GC	15
Ion chromatography	5

[a] The 20% of samples undifferentiated by the techniques in Table 1.

tor and generic) gave FTIR spectra too similar to allow judgments, and TGA scans had to be performed.

Data Evaluation

When the analyst completed the work on the sample, the sample was resealed and returned to the sample custodian. The worksheet, physical data, and instrumental spectra were forwarded to the team leader. The team leader held the sample worksheet until its mate was received. At that time, the physical data and instrumental spectra were reviewed. The team leader then labeled the pair as Status 1 (significantly different, no further work required) or Status 2 (similar, needs further work).

If the conclusion was "significantly different, no further work required," the samples were marked NAI (No Action Indicated), and the status was changed from dash (–) to 1. Approximately 80% of the samples fell into this category. If the conclusion was "similar, needs further work," the batch formula was requested from the Division of Generic Drugs in Washington, DC, and the status was changed from dash (–) to 2. Approximately 20% of the samples fell into this category.

The decision of whether samples were Status 1 or Status 2 was made by the team leader in conjunction with 2 laboratory supervisors.

Table 1 shows the percentage breakdown of the techniques used for making the determination "significantly different, no further work required." Approximately 80% of the samples were eliminated from further work using these techniques.

Thermogravimetric analysis proved to be the most useful technique for determining differences in formulations. Approximately 60% of the samples could be eliminated from further work using only this technique. XRPD is not recommended as a technique to differentiate these samples.

NMR was also used on a limited number of samples without success.

We updated the database by adding the rest of the sample information to the data fields. The Wang office management system was accessible to all managers, and managers could query the system about the status or other information on any sample.

In-depth Follow-up Examination and Data Evaluation

When the batch records were received for a pair (generic and innovator) of Status 2 samples, the records and samples were assigned to an analyst for further testing. The batch records were reviewed for differences, which formed the basis for additional testing. A variety of tests were performed, ranging from simple chemical tests for sulfate ion to complex chromatographic procedures. In some cases, corn starch was the difference in the formulations, and a polarizing microscope was used to confirm the presence or absence of starch in the samples. In other cases, magnesium stearate was targeted, and IC was used to test for magnesium ion. GC was used as the qualitative/quantitative method of analysis to determine stearic acid and lactose.

Certain formulations appeared to be identical. In this case, an impurity profile by LC was used to examine the active ingredient and related substances (3). When the formulations were tested in this manner, the liquid chromatograms of the active ingredients sometimes exhibited different impurities, thus showing different sources of the active drug material.

Table 2 shows the percentage breakdown of the techniques used for differentiating the innovator-generic samples that were initially marked Status 2, "similar, needs further work." Some formulations appeared to differ from the approved batch formula and are being investigated further.

When the additional work was completed, the samples were reviewed by the team leader and the laboratory supervisors. If the conclusion was that the samples were "significantly different, no further work required," the samples were marked NAI, and the status was changed from 2 to 7. Status Code 7 identified samples found significantly different only after additional testing and differentiated them from Status 1 samples (those found significantly different after initial FTIR and TGA examinations). Data could then be kept on how many and which samples had undergone additional testing and were found different. No samples required further testing past the Status 2 category.

Summary

In conclusion, our laboratory learned a great deal about analysis of drug formulations for excipients. The TGA and FTIR scans of powdered formulations showed many sample pairs were significantly different and quickly reduced the number of formulations requiring further work. Many simple chemical and colorimetric tests could be performed on what appeared to be a relatively complex mixture of ingredients. Chromatographic tests (GC, LC, etc.) could also be used to allow definite conclusions about the formulations. The polarizing microscope proved an invaluable tool

to differentiate formulations. Before this experience, our examination of drug samples consisted of analyzing the formulations for the active ingredients; during examination for fraud, however, spectral, chromatographic, or chemical analysis of excipients predominated.

No direct evidence of fraud was found, but inconsistencies were found in batch formulas submitted to the Division of Generic Drugs. FDA is setting up programs for further investigations.

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TECHNICAL COMMUNICATIONS

C18 Extraction of Atrazine from Small Water Sample Volumes

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A method was developed to detect atrazine at a 0.05 ppb detection limit with a 10 mL sample volume. The herbicide is extracted from 10 mL water with C18 solid-phase extraction and eluted into a reduction vessel with ethyl acetate. The sample is then evaporated to dryness under a stream of nitrogen and redissolved for injection into a gas chromatograph/mass spectrometer. Terbuthylazine is used as internal standard to compensate for extraction process variables. Standard curves have consistently shown correlation coefficients of 0.99 or better. Duplicate samples show a variance of 5% or less. Twelve samples can be extracted in about 1.5 h with <10 mL organic solvent and minimum glassware.

Atrazine (2-chloro-4-isopropylamino-6-ethylamino-*s*-triazine, CAS 1912-24-9) is a widely used triazine-based herbicide. A method was needed to determine very low concentrations of aqueous atrazine quickly, easily, and inexpensively in the presence of much larger concentrations of other organic compounds.

Other methods for the C18 solid-phase extraction of atrazine were proposed (1–7). Most of these methods require 200–1000 mL sample. Although Junk and Richard (7) studied the extraction of small sample volumes, a dedicated, long-term study has not been made. We have modified the sample handling procedure to allow the use of 10 mL sample. This modification allows the use of a 12-position vacuum manifold and drying attachment, and it decreases the volume of sample and the time required for extraction.

Most of the available methods use d^5-atrazine as an internal standard. We elected to use terbuthylazine (2-chloro-4-*tert*-butylamino-6-ethylamino-*s*-triazine, CAS 5915-41-3), which allows multiple ion fragments to be used for identification and interpretation of concentration. Terbuthylazine is a triazine-based herbicide that is closely related to atrazine; however, it is not licensed for use in the United States, nor has it been detected in samples taken from the area under study.

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Terbuthylazine is similar to atrazine in structure, melting point, and solubility, and it is available from Ciba-Geigy or the Pesticide Repository of the Environmental Protection Agency (EPA).

Experimental

Equipment and Chemicals

Atrazine (99.9%), C18 SPE cartridges (1 mL volume, 100 μg C18), and a 12-port vacuum manifold with drying attachment were obtained from Supelco (Bellefonte, PA 16823). Terbuthylazine (99.9%) was obtained from the EPA Pesticide Repository and Ciba-Geigy.

Certified ethyl acetate and methanol were obtained from Fisher Scientific Co. (Fair Lawn, NJ 07410). Water was deionized and distilled in glass in a laboratory far removed from the sample handling site. Nitrocellulose filter circles (47 mm diameter, 0.45 μm pore size, Schleicher & Schuell, Keene, NH 03431) were used in the filtering step.

Chromatograms were obtained on a Model 5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA, 94303), equipped with a 5970 mass selective detector and a 30 m SPB-1 (Supelco) cross-linked dimethylsiloxy capillary column (0.25 mm id, 0.25 μm coating thickness).

Standard Curve

Standard solutions were made by dissolving 25 mg atrazine in 25 mL ethyl acetate, and dissolving 1 mL of this preparation in 100 mL methanol to make 10 mg/L stock standards. Then, 1 mL stock standard was diluted to 1000 mL with distilled water to give 10 μg/L (10 ppb) working standards. Solutions of terbuthylazine were prepared in a similar manner, and the 10 ppb terbuthylazine solutions were diluted to 1 + 9 to give 1 ppb terbuthylazine internal standards.

From these working solutions, atrazine standards in the 0–16 ppb range were prepared, all containing 1 ppb terbuthylazine.

Sample Collection

Samples were gathered in cleaned, 500 mL amber glass bottles via a suction system. A No. 3 rubber cork with 2 No. 6 holes bored in it was inserted into the neck of the bottle, and

Table 1. Extraction efficiencies[a]

No.	Standard	Extraction
1	1856416	1339000
2	1747167	1057917
3	2125778	1226402
4	1841915	1134247
5	1673651	1202682
6	1668916	1567013
Mean	1818974	1254544
RSD[b]	0.08542	0.13073

[a] "Standard" is 5 μL 2.0 ppm atrazine injected directly; "Extraction" is 10 mL 1 ppb atrazine injected after extraction.
[b] Relative standard deviation.

two 1/4 in. (6.35 mm) diameter stainless steel tubes were inserted through the holes. A short polyethylene tube connected to a hand-powered suction pump (Black & Decker "Jackrabbit") was attached to one of these tubes, and the other tube was connected to a 30 ft (10 m) polyethylene tube weighted for lowering into the wells. The pump was used to draw air out of the bottle, causing the well water to be lifted up into the tube and flow into the bottle without coming in contact with the pump itself. After collection, the bottles were returned to the laboratory for analysis. Sediment in the samples was allowed to settle over 24 h under refrigeration (ca 5°C). Samples were then filtered through 0.47 μm pore membrane filters under suction. The internal standard was added by mixing 10 mL 10 ppb terbuthylazine with 90 mL filtered water sample in 100 mL volumetric flasks.

Extraction and Injection

Solid-phase extraction packs were conditioned by washing with 3 syringe volumes of ethyl acetate, 1 wash with methanol drained to a depth of ca 1 cm and 1 wash of distilled water drained to ca 2 cm. Then, 10 mL sample was drawn into a 10 mL disposable serological pipet and the tip was pressed into the end of the cartridge. This apparatus was allowed to elute completely under light suction (5–10 min). Dry N_2 was drawn through the cartridge under vacuum for 10–15 min to remove residual water. Samples were then eluted with two 250 μL portions of ethyl acetate (2 smaller aliquots produced better results than one 500 μL extraction). The eluant was collected in reduction vessels made by pulling points in 10 mm normal wall pyrex tubing and sealing the points. Dry nitrogen was then blown into the reduction vessels until all of the ethyl acetate evaporated, or the vessels were allowed to stand until all of the ethyl acetate evaporated.

The residue was redissolved with 50 μL ethyl acetate by wetting down the reduction vessel walls and waiting 15 min. Dry nitrogen was blown into the reduction vessel to reduce the solution volume to 5–7 μL, which was drawn into a 10 μL syringe and injected into the gas chromatograph/mass spectrometer. By allowing the sample to go to dryness and redissolving the residue, the amount of analyst involvement in the process is greatly reduced, thus allowing more samples to be run in the same amount of time.

To test extraction efficiency, a 1 ppb standard solution was extracted in the normal manner, and the effluent was extracted a second time. No atrazine or terbuthylazine was found in the eluant from the second cartridge upon normal elution. Elution efficiency was tested by extracting and eluting a 1 ppb standard solution from a cartridge and eluting the cartridge a second

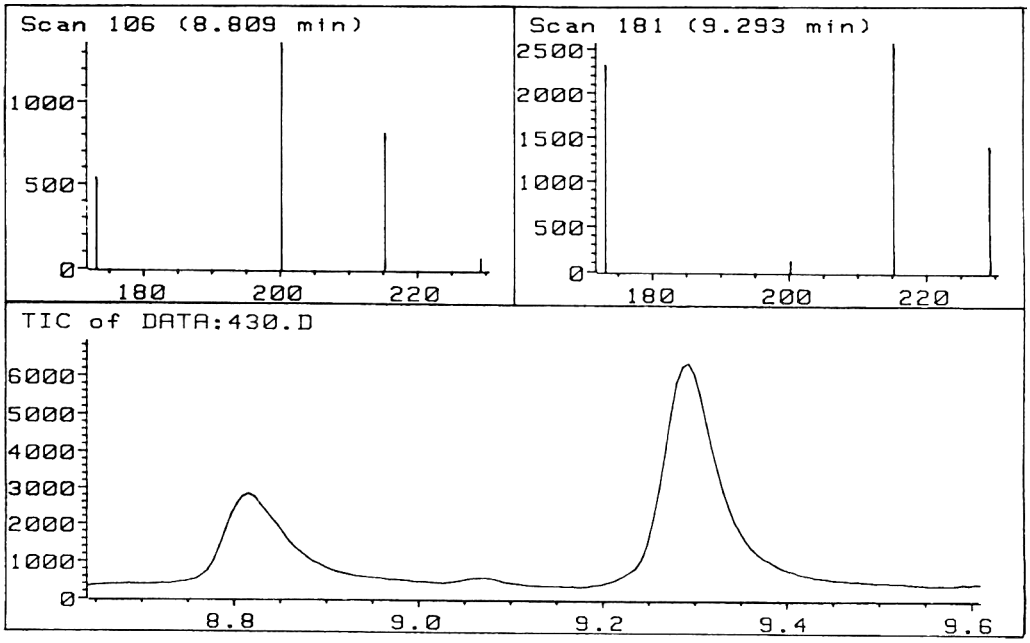

Figure 1. Ion abundance patterns for atrazine (top left) and terbuthylazine (top right) (ion counts vs atomic mass units) and total ion chromatogram (bottom) (total ion count vs time in minutes) for analysis of atrazine with terbuthylazine as an internal standard.

Table 2. Standard values for atrazine calibration curve over 120 days

Concn, µg/mL	No. of samples	Av. ATZ/TBZ[a]	SD	RSD
0.000	2	0.0285	0.0015	0.0526
0.100	4	0.1243	0.0218	0.1757
0.200	12	0.2036	0.0510	0.2502
0.300	11	0.3187	0.0549	0.1723
0.400	13	0.4786	0.0608	0.1270
0.800	9	0.9439	0.0558	0.0591
1.600	6	1.9438	0.0631	0.0324

[a] Average of ratio of peak areas for atrazine (ATZ)/terbuthylazine (TBZ).

time. No atrazine or terbuthylazine was found upon normal eluant treatment of the second elution.

Actual extraction efficiency was obtained by comparing the peak areas for 10 mL of 1 ppb standard extracted by the standard procedure and for 5 µL of 2 ppm atrazine solution in ethyl acetate injected without extraction. Each of these injections should contain 10 ng atrazine; thus, with 100% efficiency, the integrated areas should be equal. Results of these injections are shown in Table 1. Extraction efficiency was found to be ca 69% with a 9% variability. Variability of extraction efficiency is compensated by the use of the internal standard.

Sample Analysis

Samples were analyzed by gas chromatography/mass spectrometry, with injector and detector both set at 250°C. Oven temperature was set at 50°C during injection to cold trap the injected sample, and the following temperature program was used. Initial oven temperature of 50°C, hold for 1 min, raise temperature at 70°C/min to 200°C, raise temperature at 2.5°C/min to 230°C, raise temperature at 70°C/min to 290°C and hold for 4 min, and cool oven to 50°C in preparation for the next sample. Under this temperature program, atrazine will elute between 8.5 and 9.5 min and terbuthylazine will elute ca 45 s later (Figure 1). The final increase in temperature is used to elute high-boiling compounds present in the complex sample matrix.

Selected ion monitoring was used to detect the small amount of triazine against the large background of other extracted compounds. This method allows observation of only those ions that are most prevalent in the mass spectra of the herbicides. The ions monitored were *m/z* 173.15, 200.20, and 215.25 (the most abundant fragments of atrazine) and *m/z* 229.25 (the molecular ion of terbuthylazine). Because terbuthylazine has fragments of *m/z* 200.20 and 173.15, these masses also appear in the terbuthylazine chromatographic peak. Low resolution mass spectra were used to enhance peak size. Extending the allowable difference between target mass and observed mass from 0.35 to 0.45 increased the sensitivity of the detector at the expense of accurate mass detection.

Results and Discussion

A standard curve was obtained by injecting extracts from solutions having known concentrations of atrazine and terbuthylazine. The range of the standard curve actually used depended upon the expected range of samples; during March a range of 0–0.4 ppb was sufficient, whereas in July the range was extended to 1.6 ppb to account for the higher sample concentrations. In all cases, at least 4 standards were taken.

Table 2 represents the combination of all data from 16 separate standard curves taken between April 15 and August 17, 1989. This composite curve has a correlation coefficient of 0.9946, and the correlation coefficient in each of the standard curves is 0.99 or better.

Twelve samples can easily be processed in 1.5 h. With a larger or multiple extraction apparatus, several sets of 12 could be processed simultaneously. With an autoinjector, 50 samples/day is not unreasonable; it requires approximately 3 h of preparation, including standardization. In the course of our study, 1000 samples were determined in 18 months; less than 10 L each of ethyl acetate and methanol were used.

We believe that this method for atrazine greatly reduces both the time and the solvents needed to extract the samples. The use of small sample volumes (10 mL) allows for easier sample gathering and storage. The elimination of solvent extraction reduces the amount of solvent used and the amount that is released into the environment.

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TECHNICAL COMMUNICATIONS

Detection of Beet Sugar Adulteration of Orange Juice by Liquid Chromatography/Pulsed Amperometric Detection with Column Switching

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A recently reported method for the quantitation of extraneous beet sugar in orange juice is based on the monitoring of unique oligosaccharides present in beet medium invert sugar (BMIS). Modifications are reported here that expedite the method and render it better suited for monitoring a large number of samples on a routine basis. A switching valve that allows the simple sugars to go to waste after the first column prevents overloading of the analytical column and detector. Furthermore, the retention and column re-equilibration times were significantly reduced. Results of spiking studies of BMIS in orange juice are reported. The estimated limit of detection is 5% BMIS, with a coefficient of variation of 0.1%.

According to recent publications (1, 2), extraneous sugar, specifically beet medium invert sugar (BMIS), was detected in orange juice by liquid chromatography (LC) with electrochemical detection. The utility of the method derives from the fact that the same degree of sucrose inversion produces similar amounts of specific oligosaccharides (Cancalon, manuscript in preparation). The analytical methodology involves established principles of anion-exchange separation of saccharides on a quaternary amine-type resin column (CarboPac PA1, Dionex Corp., Sunnyvale, CA) with sodium hydroxide (pH > 12) as the eluant (3). BMIS was detected by using a pulsed amperometric detector, in which the sugars were oxidized at a low positive potential (50 mV) on the surface of a gold electrode (4). Distinguishing features of the reported method include the use of dual chromatographic columns that provide enough theoretical plates to resolve the oligosaccharides from the major sugars: glucose, fructose, and sucrose. A sodium acetate gradient was used to effect some control over the elution times. Postcolumn addition of 0.3M NaOH was used to maintain a stable base line. Initial trials in our laboratory corroborated the results obtained by Low and Swallow (1). The method was sensitive enough to detect BMIS in orange juice when as little as 5% of the juice was replaced by beet medium invert of the same °Brix.

We report here a modification of the method that reduces the analytical time by 50%, without any sacrifice in information or detection limits.

Experimental

Sample Preparation

Sample preparation was essentially the same as described by Low and Swallow (1) as SP2, with slight modification. Orange juice samples, produced at the Florida Department of Citrus, were diluted 2-fold with LC grade water to ca 5.5 °Brix, and 25 mL diluted sample was centrifuged 15 min at 10 000 × *g* in a refrigerated centrifuge (Sorvall RC58, Newport, CT). Then, 10 mL supernatant liquid was passed successively through a 2 mL bed of 100–200 mesh H^+-form resin (Dowex AG50W-X8), and a 2 mL bed of 100–200 mesh formate-form resin (Dowex AG 1-X4, Bio-Rad, Richmond, CA). The first 2.5 mL eluant was discarded, and the next 5.0 mL was collected. The collected sample was then passed through a Sep-Pak C18 cartridge (Millipore Co., Milford, MA), which had been conditioned with 2 mL methanol, followed by 5 mL water. The first 5 drops were discarded, and the remaining sample was finally filtered through a 0.45 μm syringe-type nylon filter (Acrodisc, Gelman Sciences, Inc., Ann Arbor, MI). Pure beet medium samples were obtained from Holly Sugar (Hereford, TX) or Amalgamated Sugar Co. (Kansas City, MO) or produced in our laboratory from pure beet sucrose. They were also diluted to 5.5 °Brix, and prepared in the same manner as the juices. Adulterated juice samples were prepared by combining BMIS and pure orange juice of the same °Brix by weight.

Liquid Chromatographic Conditions

The LC system consisted of an Ultra WISP Model 715, a Model 625 LC pumping system, and a Model 464 metal-free electrochemical detector (Waters, Milford, MA). The settings for the detector were as follows: E1 = 50 mV, T1 = 12 cycles;

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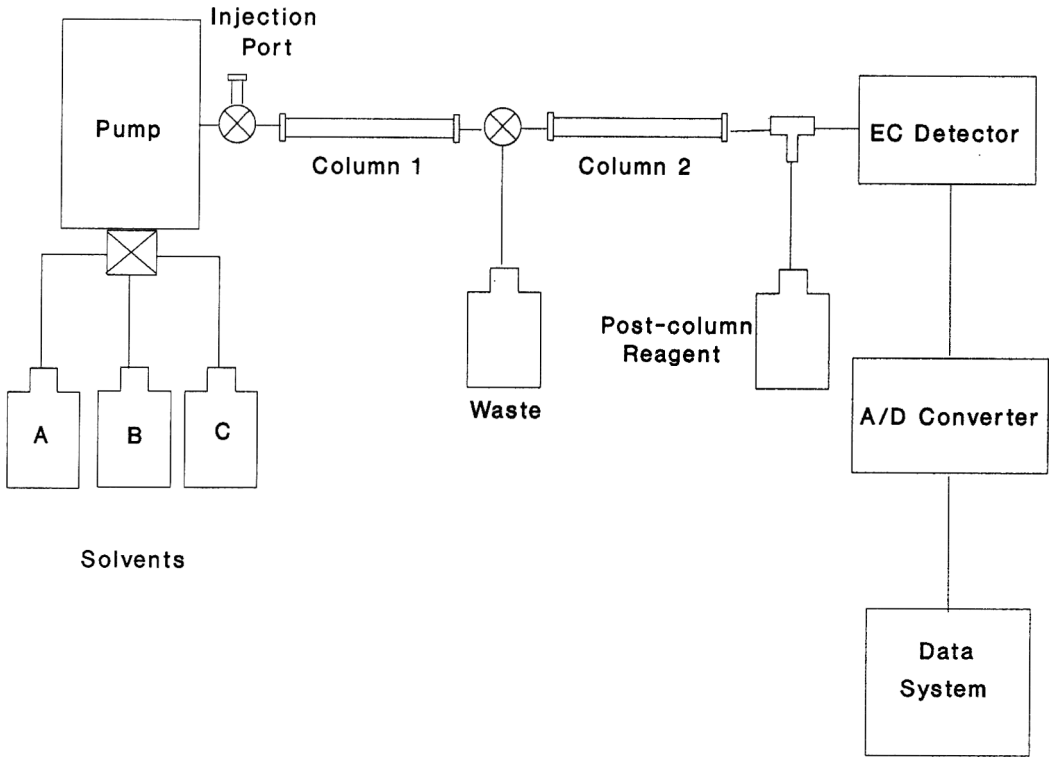

Figure 1. Schematic of the beet sugar analysis system. LC conditions are described in the text.

E2 = 800 mV, T2 = 12 cycles; E3 = – 600 mV, T3 = 30 cycles; I range = 50 μA. Control of the system and data acquisition were performed with a Waters 820 Maxima 386SX work station. The columns (Dionex Corp., Sunnyvale, CA) consisted of an ATC-1 anion trap, a CarboPac PA1 guard column, and 2 CarboPac PA1 analytical columns (Figure 1). A Valco (Houston, TX) valve with electric actuator was used for column switching. A Bio-clean self-flush pump (Scientific Systems, Inc., College Station, PA) fitted with a pulse dampener (Fisher, Springfield, NJ) was used for postcolumn addition of 0.3M NaOH.

The LC mobile Phase A was 0.1M NaOH, prepared by dilution of carbonate-free 50% NaOH (w/w) with LC-grade water, which was filtered (0.45 μm) and sparged with helium before use. Mobile Phase B was 0.1M NaOH containing 0.2M sodium acetate. Mobile Phase C and the postcolumn reagent were 0.3M NaOH. Solutions were prepared to minimize atmospheric CO_2 absorption and were continuously sparged with helium at 5 mL/min after preparation. The linear gradient was as follows: 0–4 min isocratic at 75% A, 25% B; 4–20 min linear gradient to 100% B; then 100% B for 10 min followed by 100% C for 60 min; finally re-equilibration under the initial conditions for 30 min. The conditions for the concave gradient were as follows: initially 80% A, 20% B; then 0–20 min concave gradient number 7–70% B; 10 min at 70% B followed by 100% C for 60 min and 30 min of equilibration under initial conditions.

The LC system (Figure 1) incorporated a switching valve in line between the 2 columns and steeper gradient; 50 μL was injected for each analysis. The valve was set to elute mono- and disaccharides from Column 1 to waste within the first 10 min, at which time the switching valve was activated to elute the oligosaccharides onto the second column and the detector.

Quantitation

Chromatograms of pure BMIS and adulterated juice are shown in Figures 2 and 3. Five oligosaccharide peaks were monitored for quantitation. Standard curves were established by adding known amounts of BMIS to pure orange juice. In most cases, Peaks 1–4 were integrated and the sum of the peak areas was plotted as a function of BMIS concentration (Figure 4). The main graph of Figure 4 (0–100% BMIS) was established by using the linear gradient procedure, and the inset graph (0–30% BMIS) was established by using a concave gradient developed later. This may, in part, explain the different responses obtained for similar BMIS concentrations. To take into account variations in retention time and in detector responses, new calibration curves were run with each group of samples. Quantitation was also performed by integration of Peak 5 alone (Table 1).

Results and Discussion

The major disadvantage of the earlier reported method (1, 2) was the length of time required for analysis, approximately 3 h per sample, in addition to the sample preparation. An injection volume large enough to detect the oligosaccharides overloaded the columns and detector with respect to the major sugars. A shallow gradient was necessary to provide sufficient time for the detector signal to return to base line before the oligosaccharides were eluted.

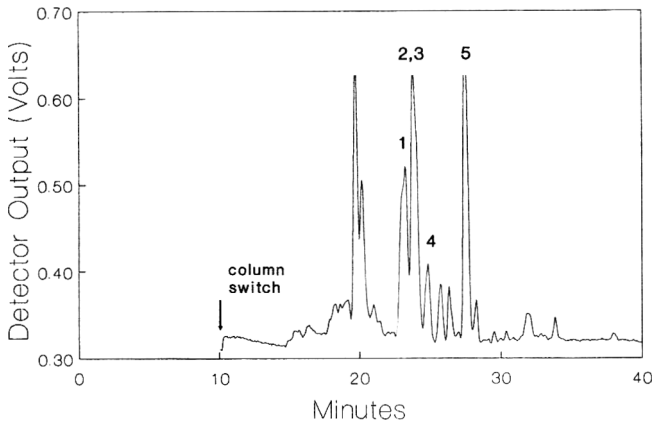

Figure 2. Chromatogram of pure beet medium invert sugar (BMIS) using the linear gradient. Peaks labeled 1–5 are mostly due to sucrose inversion-generated oligosaccharides and were used for quantitation.

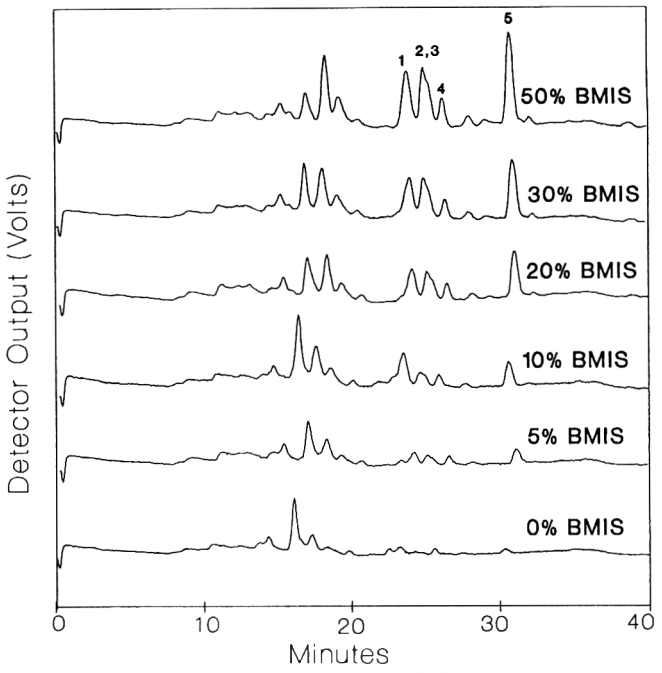

Figure 3. Series of chromatograms of orange juice containing varying levels of BMIS (concave gradient).

Because of a need to use this methodology to screen commercial juice products on a routine basis, we explored certain modifications in an effort to expedite the analysis. Although retention times were remarkably stable under the reported chromatographic conditions (1, 2), the method gave us little elution control for experimentation. In addition, we thought that some analytical precision could be gained by forcing the peaks of interest to elute earlier, i.e., during the course of the gradient. Our goal was also to minimize the possibility of accelerated column deterioration due to repeated injections of high levels of the simple sugars, as well as other deleterious compounds remaining in the prepared juice samples.

When the modified chromatographic conditions reported above were used, the peaks of interest eluted between 23 and 30 min, compared to more than 65 min under the conditions described by Low and Swallow (1). Note that the 4 numbered peaks in Figure 2 do not necessarily correspond to the 4 peaks described by Low. The oligosaccharides have not yet been identified. However, Swallow et al. (2) stated that their degree of polymerization is less than 4. Although the present system does not separate according to molecular weights, we have seen that the peaks under consideration have retention times between those of raffinose (18.1 min) and maltotriose (27.2 min), which appears immediately before Peak 5. Maltohexaose, with a retention time of 32.7 min, is released several minutes after the last BMIS peak. These preliminary results confirm that the BMIS oligosaccharides are likely to be trisaccharides.

In orange juice spiked with BMIS, the 4 peaks tend to elute as a fused "band," particularly at high BMIS concentrations. Somewhat better resolution was obtained by modifying the acetate gradient and substituting a concave gradient (Waters 625, Curve 7) between 20 and 70% mobile Phase B (Figure 3). In either case, summation of the Peak Areas 1–4 provided an excellent estimation of the juice BMIS content (Table 1). The detector response was linear between 0 and 100% BMIS (R^2 = 0.995), and at concentrations larger than 5% BMIS, the standard error was less than 10% of the calculated value.

Pure juices contain small amounts of oligosaccharides that have similar retention times. These compounds appear to be produced by a low level of yeast activity and are responsible for the non-zero intercept of the calibration graphs (Figure 4). Because the level of fermentation varies among juices, these endogenous oligosaccharides are responsible for the limitation in the accuracy of the method at low BMIS concentrations. It should be pointed out that in orange pulp wash, materials co-eluting with Peaks 1 and 4 are more abundant than in pure orange juice and may represent up to 8% BMIS when expressed as beet sugar. We have indications that these oligosaccharides are not specific to pulp wash but may reflect a higher level of fermentation in this product.

The overestimation of BMIS in juices containing pulp wash can be averted by using Peak 5 for the quantitation (Table 1).

Table 1. BMIS measurements[a]

Juice type	BMIS added, %	Measured BMIS concn, % Four peak method		Single peak method	
OJ	0	0.7 ± 0.6	(25)	0.8 ± 0.7	(17)
OJ	1	2.1 ± 1.3	(6)	1.6 ± 1.1	(3)
OJ	2.5	2.3 ± 0.7	(6)	2.1 ± 0.8	(6)
OJ	5	4.8 ± 0.6	(6)	4.5 ± 0.6	(6)
OJ	10	10.6 ± 0.8	(6)	10.5 ± 0.8	(6)
OJ	20	21.1 ± 0.7	(6)	20.7 ± 0.8	(6)
OJ	50	52.0 ± 1.1	(3)	49.7 ± 1.3	(3)
PW	0	4.4 ± 2.8	(3)	1.0 ± 0.4	(3)
PW	5	15.4 ± 2.0	(3)	7.2 ± 1.9	(3)

[a] Mean ± SD of %BMIS measured; (n) number of samples examined; OJ = orange juice; PW = pulp wash.

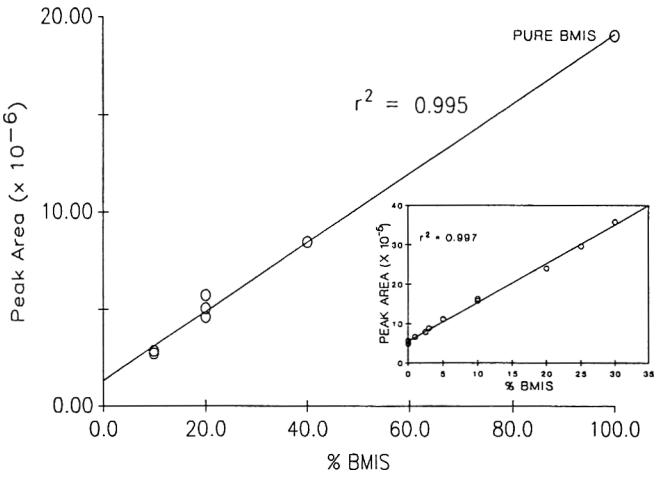

Figure 4. Correlation of peak area (Peaks 1–4) with BMIS concentration. Main graph: using linear gradient. Inset: 0–30% BMIS (using concave gradient), the range mostly found in commercial products.

Furthermore, careful examination of the shape of the 4 peak profiles indicates that BMIS adulteration is distinguishable from pulp wash addition.

Conclusion

The method described provides a good estimation of beet sugar addition in citrus juices and is being extensively used in our laboratory for the monitoring of commercial products. Its primary limitation is the influence of endogenous saccharides that interferes with quantitation at low BMIS concentrations. Pulp wash can also significantly alter BMIS estimation when the 4-peak area summation method is used. Monitoring of Peak 5 in these cases circumvents this problem. A correspondence between the 2 quantitation methods is desirable, particularly at low BMIS concentrations. Also, preliminary studies indicated that the extent of the inversion process is a factor that may affect the relative size of the peaks and consequently the quantitation of extraneous sugar in juices.

Acknowledgment

The BMIS standards used to generate the data presented in the main part of Figure 4 were provided by the U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition.

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TECHNICAL COMMUNICATIONS

Gas Chromatographic Determination of Dimethoate Residues in Chrysanthemums and Soil

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A gas chromatographic (GC) method was developed for determination of dimethoate residues in chrysanthemums and soil. Dry flowers, fresh flowers, and leaves of chrysanthemum and soil samples are extracted with acetone, cleaned up by coagulation, and partitioned with dichloromethane. Dimethoate is determined by GC with nitrogen-phosphorus detection. Recovery ranges of dimethoate from samples of dry flowers, fresh flowers, and leaves of chrysanthemums are 86.7–91.0% (fortified at 0.5–2.0 ppm), 88.1–93.6% (fortified at 0.036–0.721 ppm), and 83.3–96.9% (fortified at 0.5–1.31 ppm), respectively. Recovery ranges of dimethoate from soil samples are 93.0–104% (fortified at 0.125–0.327 ppm). Average recoveries of the 4 kinds of samples (n = 9) are 88.4 ± 8.94%, 91.5 ± 4.18%, 91.1 ± 4.69%, and 97.3 ± 5.1%, respectively.

Dimethoate, *O,O*-dimethyl-*S*-(*N*-methylcarbamoylmethyl)phosphorodithioate, is a widespread systemic insecticide. Chrysanthemums [*Dendranthemamorifolium* (Ramat) Tzvel] are important medicinal herbs that are planted widely in China. Dimethoate can control many kinds of pests, but it is widely used to protect chrysanthemums from aphids (*Aphis gossypii* Glover and *Pyrethrmyzus sanborni* Gilletti) and stem borer (*Phytoecia rufiventris* Gautier). To determine dimethoate residues in chrysanthemum products and keep them below the maximum residue limit, it is necessary to develop a method to determine residues in dry flowers, fresh flowers, leaves of chrysanthemums, and soil samples. Many authors have presented gas chromatographic (GC) methods to detect dimethoate residues in rice, vegetables, foods, and soil. However, no method has been published for determining dimethoate residues in chrysanthemum samples. In addition, many of the published methods for dimethoate in other substrates involve cleanup procedures with solid-phase partition columns (1–8), highly toxic solvents (2, 8–10), and derivatization procedures (9). The drawbacks of the above and other similar procedures are lengthy analysis time and effects of toxic solvents on the health of laboratory workers. In addition, the packing of cleanup columns can seriously affect the efficiency of cleanup and sometimes will require large quantities of solvents for elution (1, 2). The object of the present investigation is to develop a GC method that is simple, economic, quick, and efficient for determination of dimethoate residues in chrysanthemum and soil samples.

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Experimental

Apparatus

(a) *Gas chromatograph.*—Perkin-Elmer Sigma 2000 equipped with nitrogen-phosphorus detector (NPD). GC column: glass 1.5 m × 2 mm id, packed with 5% OV-17 on 80–100 mesh Chromosorb W DMCS. Operating conditions: nitrogen carrier, 60 mL/min; hydrogen, 8 mL/min; air, 140 mL/min; column oven, 210°C; detector, 250°C; injection port, 230°C; injection volume, 1–5 μL; chart speed, 0.5 cm/min.

(b) *Rotary evaporator.*—ZFQ85A (made in China).

(c) *Blender.*—Waring.

(d) *Concentrator.*—Kuderna-Danish (made in China).

(e) *Shaker.*—HY-2 (made in China).

Reagents and Materials

(a) *Samples.*—Supplied by Tonxian Medical Co.

(b) *Dimethoate.*—40% Emulsifiable formulation (made in China); standard, purity 99% (Institut für Lebensmittelchemiàe, Germany).

(c) *Solvents.*—All organic solvents (e.g., acetone, dichloromethane) were analytical grade, distilled in glass (made in China).

(d) *Anhydrous sodium sulfate.*—Analytical grade (made in China).

(e) *Phosphoric acid.*—85%, Analytical grade (made in China).

(f) *Ammonium chloride.*—Analytical grade (made in China).

(g) *Celite 545.*—(Fluka, Japan).

(h) *Active carbon.*—Analytical grade powder (made in China).

(i) *Coagulating solution.*—Dissolve 20 g ammonium chloride and 40 mL 85% phosphoric acid in 360 mL distilled

Table 1. Recovery of dimethoate from dry flowers, fresh flowers, leaves of chrysanthemums, and soil

Sample	Added, ppm	Rec., %[a]	SD
Dry flowers	2.00	87.5	8.24
	1.00	86.7	10.8
	0.500	91.0	7.78
Av.	—	88.4	8.94
Fresh flowers	0.721	92.7	3.92
	0.0721	88.1	4.03
	0.0360	93.6	4.60
Av.	—	91.5	4.18
Fresh leaves	1.31	93.2	1.91
	1.00	83.3	11.3
	0.500	96.9	0.85
Av.	—	91.1	4.69
Soil	0.327	104	12.9
	0.250	93.0	1.00
	0.125	95.0	1.41
Av.	—	97.3	5.10

[a] Values are average of triplicate analyses at each fortification level.

water (concentrated solution). Concentrated solution usually may be diluted 5- to 10-fold (diluted solution) in use.

Determination of Dimethoate

(a) *Fresh leaves, fresh flowers, and dry flowers.*—Weigh 5 g fresh leaves or dry flowers or 20 g fresh flowers into blender bowl. Add 100 mL acetone and blend 2 min at high speed. Filter through layer of Celite 545 (add a thick layer of active carbon on the top of Celite 545) in Buchner funnel. Apply vacuum to suck dry. Return filter cake to blender bowl. Blend with 100 mL acetone and filter as before. Combine acetone extracts into round-bottom flask and evaporate acetone to ca 5 mL in a rotary vacuum evaporator in 40°C water bath. Add 10 mL concentrated coagulating solution, 40 mL distilled water, and a little Celite 545. Mix and let solution stand for 10 min. Filter through thin layer of Celite 545 in Buchner funnel. Rinse with a little diluted coagulating solution. Apply vacuum to suck dry. Transfer filtrate to 250 mL separatory funnel. Add 100 mL distilled water and 5 g sodium chloride; extract 3 times with 50 mL portions of dichloromethane. Filter dichloromethane extracts through anhydrous sodium sulfate into Kuderna-Danish concentrator and evaporate to ca 1 mL under vacuum on 40°C water bath. Add two 10 mL portions of acetone to evaporate to ca 1 mL. Adjust to suitable volume for GCNPD determination.

(b) *Soil.*—Weigh 20 g soil (wind dried and sieved through 40 mesh screen) into 250 mL Erlenmeyer flask. Add 100 mL acetone and shake 1 h in a vibrating machine. Filter through layer of Celite 545 in Buchner funnel. Apply vacuum to suck dry. Return filter cake to flask, add 100 mL acetone, and repeat procedure once. Combine acetone extracts. Follow procedure for plant tissue.

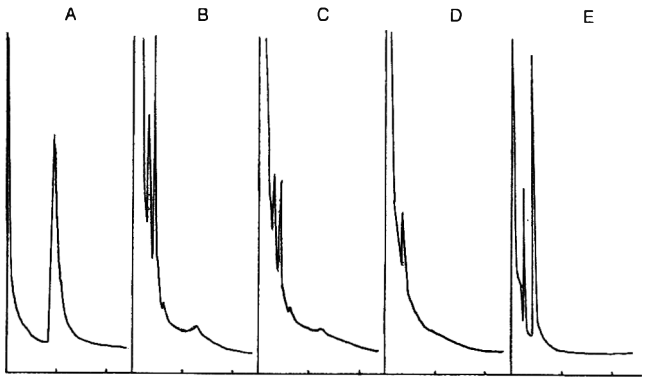

Figure 1. Typical chromatograms of dimethoate: (A) 1.3 ng dimethoate standard; (B) extract equivalent to 10 mg dry flowers; (C) extract equivalent to 40 mg fresh flowers; (D) extract equivalent to 10 mg leaves; (E) extract equivalent to 40 mg soil.

(c) *GC analysis.*—Use 5% OV-17 column and peak height and external standardization for determination of dimethoate. Under given operating conditions, retention time of dimethoate is 2 min and 43 s.

(d) *Fortification procedure.*—For recovery study, standard solution of dimethoate (using acetone as solvent) was directly added to untreated samples, mixed, and allowed to stand for ca 1 h before extraction solvent was added.

Results and Discussion

With this method, recovery ranges of dimethoate from 4 kinds of samples were 86.7–91.0% (fortified at 0.5–2.0 ppm) for dry flowers, 88.1–93.6% (fortified at 0.036–0.721 ppm) for fresh flowers, 83.3–96.9% (fortified at 0.5–1.31 ppm) for fresh leaves, and 93.0–104% (fortified at 0.125–0.327 ppm) for soil. Average recoveries (n = 9 for each type of sample) were 88.4 ± 8.94%, 91.5 ± 4.18%, 91.1 ± 4.69%, and 97.3 ± 5.1%, respectively (Table 1). Typical chromatograms of standard and untreated samples are shown in Figure 1.

Minimum detector response was to 0.1 ng dimethoate. Minimum theoretical detectable limits of the method for dimethoate in 4 kinds of samples were 0.005–0.01 ppm for 20 g samples of fresh flowers and soil and for 5 g samples of dry flowers and fresh leaves. The final concentrated volume for detection was 1 mL, and 2 μL aliquots were injected.

In the present study, coagulation was successfully used to precipitate fat-soluble pigments and impurities in analytical samples. In contrast with column chromatographic methods (1–8), the coagulation method is simpler and easier. It overcomes the drawbacks that the nonuniformity of packed columns causes. The present method compares well with those of Lee and Westcott (1) and Ferreira and Tainha (2). The present method is also economical in its use of solvents and reagents. The coagulation method has been widely used to determine carbamate pesticides in plants, animals, and soils (11–14). The present method differs in its use of acetone and dichloromethane as extraction solvents instead of more highly toxic

substances such as acetonitrile (2, 8–10), which are especially hazardous in inadequately ventilated laboratories.

The method described here has been successfully used for determination of dimethoate residues in treated chrysanthemums and soil. It is simple, economic, quick, and efficient and should be applicable to other plants and crops.

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