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For Your Information

Meetings

April 16, 1998: AOAC MidAtlantic Section, Laurel, Maryland, USA. Contact: Joan Pinkas, McCormick and Co., 202 Wight Ave, Hunt Valley, MD 21031, USA, +1-407-771-7811

April 26–30, 1998: AOAC Southeast Section, Atlanta, Georgia, USA. Contact: Mike Farrow, Pesticide Enforcement Agency, Georgia Department of Agriculture, PO Box 1507, Tifton, GA 31793, USA, +1-912-386-3147

May 5–6, 1998: AOAC Northeast Section, Newton, Massachusetts, USA. Contact: Dana A. Krueger, Krueger Food Laboratories, Inc., 24 Blackstone St, Cambridge, MA 02139, USA, +1-617-876-9118

June 3–4, 1998: AOAC MidAtlantic Section, Lancaster, Pennsylvania, USA. Contact: Deborah A. Marcuson, Lancaster Laboratories, PO Box 12425, 2425 New Holland Pike, Lancaster, PA 17605-2425, USA, 1-717-656-2300

June 8–10, 1998: AOAC Midwest Section, Madison, Wisconsin, USA. Contact: John C. Walton, HES, Inc., 525 Science Dr, Madison, WI 53711, USA, +1-608-232-3308

June 11–12, 1998: AOAC Europe Section Symposium and Workshop: Quality Assurance for Computerized Laboratories—Analytical, Clinical, and Pharmaceutical Laboratories, Copenhagen, Denmark. Contact: Lorens Sibbesen, DTI, Department of Chemistry, POB 141, DK 2630 Taastrup, Denmark, +45-43-50-46-67

June 11–12, 1998: AOAC Pacific Northwest Section, Tacoma, Washington, USA. Contact: Stephen V.W. Pope, U.S. Environmental Protection Agency, Manchester Laboratory, 7411 Beach Dr, Port Orchard, WA 98366, USA, +1-306-871-8717

June 18, 1998: AOAC MidCanada Section, Winnipeg, Manitoba, Canada. Contact: Anja Richter, Canadian Grain Commission, 303 Main St, Winnipeg, MN, R3C 3G8, Canada, +1-204-984-7456; or Gary Lombaert, Health Canada, 510 Lagimodiere Blvd, Winnipeg, MB, R2J 3YI, Canada, +1-204-984-2088

June 28–30, 1998: AOAC Southwest Section, Corpus Christi, Texas, USA. Contact: Sara M. Williams, Office of the Texas State Chemist, PO Box 3160, College Station, TX 77841-3160, USA, +1-409-845-1121

September 13–17, 1998: The 112th AOAC INTERNATIONAL Annual Meeting and Exposition, Montreal, Quebec, Canada. Contact: Meetings and Education Department (meetings@aoac.org), AOAC INTERNATIONAL, 481 N Frederick Ave, Suite 500, Gaithersburg, MD 20877 USA, +1-301-924-7077, fax +1-301-924-7089

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September 10–14, 2000: The 114th AOAC INTERNATIONAL Annual Meeting and Exposition, Philadelphia, Pennsylvania, USA. Contact: Meetings and Education Department (meetings@aoac.org), AOAC INTERNATIONAL, 481 N Frederick Ave, Suite 500, Gaithersburg, MD 20877 USA, +1-301-924-7077, fax +1-301-924-7089

Viorica Lopez-Avila Wins 1998 Wiley Award

This year's Harvey W. Wiley Award, AOAC INTERNATIONAL's highest scientific honor, goes to a scientist whose search for new analytical methods has led not only to the boundaries of established fields like mass spectrometry, but to the conversion of a standard kitchen appliance into a powerful analytical tool.

The Wiley Award is given each year to a scientist whose career has produced significant advances in analytical methodology. The 1998 recipient is Dr. Viorica Lopez-Avila, director of California operations for Midwest Research Institute (MRI), a researcher whose hallmark traits are common to many Wiley Award awardees: a bubbling enthusiasm for a great diversity of analytical fields and a quick intellect that allows her to seek out innovative methods.

Primarily an environmental analyst specializing in extraction and sample preparation, Lopez-Avila is nationally recognized for pioneering the use of

microwave-oven technology to simplify complex extractions.

These microwave-assisted methods work in much the same way as everyday microwave ovens: using microwave radiation to heat water or other solvents capable of absorbing microwave energy. But instead of cooking vegetables, the goal is to superheat a solvent to dramatically speed up extraction processes.

Microwave heating, for example, may be used to break down plant cells to help release pesticides or other organic analytes. Another application is for extracting organic compounds from soil samples. The process uses closed, pressurized vessels that allow heating the solvent to far above its normal boiling point, at pressures on the order of 150 pounds per square inch. "We operate somewhere around 110 to 120°C," she says, adding: "The first time we did it, we had an explosion."

But once the process was perfected, she says, she found that once-lengthy extractions took only a few minutes.

One use for microwave heating, she said, is with a standard reflux procedure called the Soxhlet extraction. As conventionally performed, she says, this process "takes hours and hours—usually overnight." It calls for boiling the solvent and condensing it so it percolates through the matrix over and over. "But with the microwave," she says, "it takes 5 to 10 minutes to reach 110 to 120°C, then all you have to do is heat it for 10 minutes."

The process works with a variety of solvents, including methylene chloride/acetone and hexane–acetone mixtures, methyl tertiary–butyl ether, or toluene–methanol mixes. But it is particularly valuable with aqueous buffers, because samples extracted from such buffers are immediately ready for immunoassay testing (typically performed in aqueous solutions). Not surprisingly, Lopez-Avila is a leader in combining immunoassay methodologies with microwave extractions.

She became interested in analytical chemistry in the mid-1970s, as a research assistant at Massachusetts Institute of Technology, shortly after emigrating from her native Romania. Her degree was on the interface between chemistry and chemical engineering, in the engineering technology of organic substances. "I wasn't much interested in theory," she says. "I was more into applied chemistry."

This led her to join the Chemical Engineering department, where Ronald Hites, now a distinguished professor of public and environmental affairs at Indiana University, was pioneering the use of GC/MS methods for environmental pollutants. "I owe it to him for getting me into the field," she says. From the moment she discovered that her interests "fitted perfectly" with Hites' work, she says, "I never looked back."

In 1979, Lopez-Avila was hired by MRI, working 2 1/2 years in Kansas City as a senior chemist. Then, a Mountain View, California, USA, firm called Acurex gave her a dream job: working as a contractor for the U.S. Environmental Protection Agency's National Exposure Research Laboratory in Las Vegas, Nevada, USA. Beginning as principal investigator and later graduating to program manager, she helped develop more than 20 methods for pesticides, petroleum hydrocarbons, and other environmental pollutants.

In 1990, the Acurex facility was sold and converted from a research facility to a testing laboratory. Lopez-Avila returned to MRI, with her EPA contract. The split was friendly, allowing her to remain in California, subletting lab space from her former employer. "We're friends," she says. "We kind of have invisible doors. They're there but we never lock them."

At the peak of her new operation, from 1991–1995, she had as many as 17 employees, but that ended in 1995, when Lockheed underbid MRI for the EPA contract.

Lopez-Avila remains in Mountain View, but with a much different role, wearing the hat of "scientific advisor." In this capacity, she says, "I'm always on the lookout for new technologies." It's a dream job for an innovative thinker, requiring her to seek out small companies with interesting technologies that can be developed for new applications for MRI's clients.

One example is a process called membrane introduction mass spectrometry (MIMS), new for environmental chemistry. It uses a special capillary tube, made by a single U.S. vendor, which inserts into the ion source of a mass spectrometer. The tube is composed of a silicon membrane that prevents the diffusion of water molecules but not organic pollutants.

This, she says, allows the organics to be immediately introduced into the ion source of the mass spectrometer, without need for conventional extraction techniques, which require purging the organics from the water, trapping them on an absorbant material, thermally desorbing them from the trap and separating them by gas chromatography. "The standard technique takes about 45 minutes and is very complicated," she says. "With the MIMS method, I can analyze a water sample every 5 minutes."

It's not a perfect process, she admits, because the lack of a chromatographic column makes it difficult to analyze a sample with a large array of contaminants. "However," she says, "a lot of water samples are very clean, so you can use this technology to screen. If you find a sample that's contaminated, you can then subject it to the conventional analysis."

To further expedite the method, Lopez-Avila has been working with the MIMS manufacture and Hewlett-Packard to fund the design and testing of an autosampler that will work with HP mass spectrometers.

In addition, she has a book contract to edit a compendium of field analytical methods—including the new MIMS technology.

In general, she suggests that much of the future of environmental analysis is in field technology. "Environmental methods are changing," she says. "People in the field, who do the remediation work, cannot make decisions when they have to wait 3 or 4 weeks for results. They cannot afford to extract a sample for 24 hours. They need to extract it in 10 minutes and analyze it in another 10. They want technologies they can use on site. Environmental methods are leaving the lab and moving into the field. Or, sometimes we take the lab into the field in a mobile van."

Environmental methods, she says, need to themselves become more environmentally friendly. "We need to develop methods that don't use a lot of organic solvents," she says, adding that this is one of the advantages of microwave-assisted and supercritical fluid extraction techniques.

Colleagues note that Lopez-Avila's strengths include not only her wide-ranging intellect, but also an infectious enthusiasm and winning personality. "She's very much a go-getter, very thorough, and enjoys what she does," observes Dr. Jeanette Van Emon, a research chemist at EPA's Las Vegas laboratory who worked as assignment manager on Lopez-Avila's contract.

Van Emon appreciates the practical perspective Lopez-Avila inherited from her engineering background. Lopez-Avila, she says, is good at tailoring methods to fulfill particular needs, rather than thinking: "I can go down to a part-per-trillion, so perhaps I should."

Dr. Werner Beckert, a retired EPA research chemist who worked with Lopez-Avila for 15 years, praises her unusual diligence in reporting on the progress of individual projects "practically every working day." He also hails her as a great beneficiary of the taxpayers for her ability to charm free instrumentation from manufacturers.

Lopez-Avila has also been active in AOAC, beginning in 1987, when she realized that EPA didn't have guidelines for how to do collaborative studies to verify the methods she was developing. She contacted AOAC at a time when AOAC members were mostly involved with foods and drugs, but when AOAC was actively courting environmental laboratories.

Almost immediately, she found her talents put to use, serving as general or associate referee on four simultaneous pesticide studies. The pace of her AOAC work subsequently became less hectic, but she is still involved in at least one collaborative study every year or two. Currently she is serving as General Referee on two studies: a completed one on the volatile emission potential of liquid and solid pesticides by thermal gravimetry and an upcoming study on the determination of DDT and DDE by enzyme immunoassay. She also serves on AOAC's Peer Verified Methods Committee.

Lopez-Avila attacks other interests with equal vigor. She has a 7-year-old daughter with whom she spends as much free time as she can scrounge, even leaving her lab every Friday afternoon to assist in her daughter's school. "I'm a volunteer mother in the classroom," she says. "I go on field trips. I do art projects, file classwork, copy materials—whatever the teacher needs."

She may even be helping to train the next generation of chemists. "I do experiments with the kids," she says. "During National Chemistry Week, we mixed glue and borax to make goofy putty and used colorimetric test strips to determine water hardness."

AOAC Launches Laboratory Proficiency Testing Program

AOAC INTERNATIONAL, recognized throughout the world as a leader in analytical excellence, is launching a laboratory proficiency testing program in mid-1998 to help scientists support their quality assurance programs.

AOAC is creating an international program for proficiency testing that encompasses both chemical and microbiological analysis of food and environmental samples," AOAC President Paul Beljaars stated. "With the launch of the AOAC Laboratory Proficiency Testing Program, we now offer comprehensive proficiency testing services that are in full compliance with ISO guidelines and recognized by government agencies, industry, and accreditation bodies."

Survey Gauges Market Interest

To determine the level of interest in improved and expanded proficiency testing programs, AOAC conducted a survey in the summer of 1997 that drew a higher than usual response rate.

One of the main findings of the survey was that a single, comprehensive proficiency testing program would be very attractive to subscribers. Currently, many laboratories must use two or more programs, an inconvenience which survey respondents complained is difficult to manage. Even those laboratories that are participating in several programs reported that not all of their proficiency testing needs are being met. Respondents also complained that there are analytes, organisms, and matrixes that are not included in any proficiency testing program that is readily available to all interested subscribers.

Having established that there is a need for a comprehensive proficiency testing program, AOAC leadership decided to proceed with plans to enter the field. It was felt that AOAC, as an international scientific and educational association, has a mission to promote advancements in method validation and quality measurements.

One specific advantage of AOAC participation, leadership felt, was that the program would permit laboratories to verify the performance of AOAC's *Official Methods of Analysis*, the *Peer Verified Methods*, and the *Performance Tested Methods* in their laboratory.

Growth in Contract Laboratories

AOAC expects that the program will be particularly attractive to contract laboratories because they analyze many analytes or organisms in many matrixes, and need to verify laboratory performance with the laboratory's equipment, reagents, and trained personnel for a particular method. Contract laboratories also have to satisfy clients and regulatory agencies that laboratory perfor-

mance is acceptable, and provide independent quality measures.

The number of contract laboratories is increasing rapidly as large companies outsource more of their analytical work and small startup firms that do not have the resources to do their own analytical work enter the market. Continued growth is expected in the field of contract analytical services, creating a large pool of potential subscribers.

Other types of laboratories needing proficiency testing include environmental laboratories interested in a program that is recognized and accepted by many regulatory agencies, and quality control and quality assurance laboratories using proficiency testing as an independent measure of quality.

In addition, the program is expected to appeal to large companies that need to verify laboratory performance at multiple locations, and to food processor laboratories and government laboratories.

The AOAC survey revealed particularly strong interest in a proficiency testing program for microbiological organisms. In the United States, there is currently no comprehensive microbiological proficiency testing program.

Range of Testing

In order to develop a truly comprehensive program that can provide subscribers with "one stop shopping," AOAC determined that the program must include all analytes or organisms and matrixes for which a high rate of interest has been demonstrated. These include:

- Food nutrition
- Pesticides
- Microbiological organisms
- Environmental contaminants
- Water

The program is designed so it can be expanded to respond to new needs of subscribers.

To advise AOAC on its Laboratory Proficiency Testing Program, experts have been recruited to serve on advisory task forces on microbiology, food nutrition and feed, and pesticide residues. An environmental advisory task force will be formed in the future.

User fees were set to cover the cost of operation while providing subscribers with proficiency testing services at a reasonable cost.

For further information on the AOAC Laboratory Proficiency Testing Program, contact Arlene Fox at AOAC INTERNATIONAL, 481 N. Frederick Ave, Suite 500, Gaithersburg, MD 20877-2417, USA, telephone +1-301-924-7077, fax +1-301-924-7089, e-mail: afox@aoac.org.

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Food Biological Contaminants

Comparison of SimPlate™ Total Plate Count Test with Plate Count Agar Method for Detection and Quantitation of Bacteria in Food—*D.E. Townsend and A. Naqui*

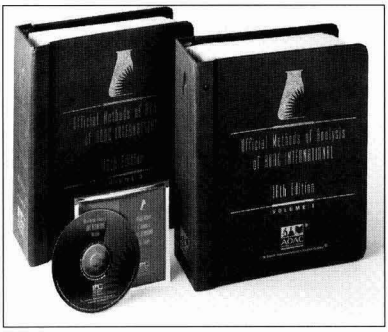
Food Composition and Additives

Stable Carbon Isotope Ratio Analysis of Honey: Validation of Internal Standard Procedure for Worldwide Application—*Jonathan W. White, Kenneth Winters, Peter Martin, and Andreas Rossmann*

Residues and Trace Elements

Comparison of Six Methods for Determining Aged Phosphine Residues in Wheat—*Sylvia E. Allen, Ren Yonglin, and James M. Desmarchelier*

A Simple System for Rapid Determination of Carbon Dioxide Evolution Rates—*Yuch-Ping Hsieh*



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Application Note 81 describes a simple, straightforward method using the Dionex IonPac AS9-HC column to determine trace levels of oxyhalides and bromide in the presence of common anions in drinking water. The IonPac AS9-HC satisfies the requirements as an optional column for U.S. Environmental Protection Agency Method 300.0(B), which allows it to be used for compliance monitoring of bromate, bromide, chlorate, and chlorite, according to the EPA Information Collection Rule. Using the method described in this Note, all of the ICR target anions can be determined by direct injection in one 25 min isocratic run and detected in a concentration range that exceeds the ICR requirements. Unlike previous IC methods, no sample pretreatment cartridges are required to remove high concentrations of common ions, such as chloride, bicarbonate, and sulfate. Detection limits and linear concentration ranges are also presented.
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SPECIAL REPORTS

Hyphenated Techniques in Thin-Layer Chromatography

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This special report deals with the critical evaluation of the present state of hyphenated techniques in thin-layer chromatography, the enumeration of possible advantages and disadvantages of the newest developments, and prerequisites for the successful application of the newest results.

Thin-layer chromatography (TLC) was developed more than 35 years ago for separation of individual molecules from complicated matrixes (1). In past decades, use of TLC techniques in separation and/or quantitative determination of organic and inorganic solutes has increased markedly (2, 3). The increase may be due to the simplicity and relatively low cost of traditional TLC methods (4), the development of new methods and instrumentation (5), and the consistent interest of chromatographers in this appealing and attractive technique (6). The latest theory and practice of contemporary TLC are compiled and discussed biannually in *Analytical Chemistry* (7, 8). The well-known drawbacks of TLC (relatively low sensitivity, uncertainty of identification of solutes) necessitated development of combined methods suitable for more precise identification and better sensitivity.

The objectives of this work are compilation and evaluation of methods coupling TLC to other analytical procedures—such as gas chromatography (GC), mass spectrometry (MS), Raman spectroscopy, Fourier transform infrared spectrometry (FTIR), square-wave stripping voltammetry, solid-phase nuclear magnetic resonance (NMR) spectroscopy, and atomic absorption spectrometry (AAS)—evaluation of advantages and disadvantages of these combined methods, and prediction of future uses of these new methods.

Traditional TLC

Traditional TLC is a rapid and simple separation method, as simple as paper chromatography but more convenient and easier to perform. A solution of sample is spotted onto the plate, and the plate is developed with a generally simple eluant. After development, the plate is dried, and the spots are detected. The advantages of traditional TLC are minimal need for instrumentation and trained personnel, and low specific cost of analysis (9). However, traditional TLC methods suffer some serious drawbacks (10). Reproducibility of retention values are sometimes poor, and the relative standard deviation of quantitative determination is frequently high and unacceptable. Traditional TLC methods not always can comply with strict requirements of up-to-date validation processes.

Because of their obvious advantages, TLC methods continue to be used in synthetic laboratories for rapid control of purity of intermediates and final products. They may also have potential as a pilot method for liquid chromatography (LC; 11, 12), and they offer a unique possibility to detect solute components remaining on the origin, which are pratically undetectable by LC.

Instrumental TLC

The position of TLC among chromatographic techniques changed with commercialization of instruments that automate practically all steps in TLC analysis. The precision and reliability of sample application (a crucial step in quantitation) have been increased markedly by various application devices (13), which make it possible to apply an accurate volume of solution on any site of the plate. Different development methods—such as linear ascending, horizontal, circular and anticircular—need some instrumentation with significant increase of the separation efficacy but their use requires more time and expertise. Automated developing instruments considerably decreased the error caused by use of simple developing chambers. They facilitate application of two- or multistep development, which markedly enhances TLC separation (14, 15). However, the efficacy of multistep gradient elution in TLC never will be higher than that of continous gradient elution in LC. Introduction of various TLC scanners combined with sophisticated softwares revolutioned quantitative determination in TLC (16). They allow more precise determination of the position of spot maximum important for theoretical studies and for solute identification; make possible determination of absorption spectra of solutes in situ, which can be different from those determined in solution; and provide the same facilities for the quantitative evaluation as softwares do for LC.

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Hyphenated Techniques in TLC

In the past decades, the future of TLC has been discussed vigorously (17, 18), with opinions ranging from extinction to renaissance. Although application and validation parameters of modern TLC are comparable with those of LC (19, 20), the inherent limitations of TLC (ambiguous solute identification and quantitation according to retention behavior and absorption spectra) can be overcome only by use of hyphenated techniques, which can be defined loosely as any method combined with TLC. Most hyphenated techniques use TLC to separate solutes. Spots are scraped, and solutes are dissolved in a suitable solvent. Solute concentrations are determined by spectrophotometry (21). Sometimes TLC is used to remove lipids from the sample, and sample constituents then are separated by GC (22). By combining 2 existing chromatographic methods in on-line mode, the efficacy of the separation or quantitation is increased. However, the same effect can be achieved by using a TLC scanner or a more sophisticated GC procedure (coupled-column GC).

In this paper we use a stricter definition of a hyphenated TLC technique: a separation process that uses one or more instruments based on theoretically different physical and/or physicochemical principles and is coupled to TLC to increase the efficacy (identification power, sensitivity, etc.) of ordinary TLC.

TLC–Various Mass Spectrometric (MS) Techniques

Here, MS is coupled to other chromatographic methods such as GC (23) and LC (24) for easier identification of individual components in natural mixtures. This hyphenated technique has found wide acceptance and application. Earliest applications of TLC–MS were limited to identification of solutes eluted from sorbent by traditional wet methods and to the use of these solutions for MS investigations. Although this procedure facilitates identification, it is time-consuming, and the danger of contamination is fairly high.

Much effort has been devoted to development of methods for determining MS spectra directly on the support. Thermally stable, volatile solutes can be analyzed after volatilization by electron impact (EI) ionization; secondary ion mass spectrometry (SIMS), fast atom bombardement (FAB), or liquid secondary ion mass spectrometry (LSIMS) can be used to study less volatile or unstable solutes. The possibility of MS scanning of TLC plates also has been investigated extensively. Volatilization of solutes from any defined part of the plates before MS can be done either with pulsed CO_2 laser or with high-intensity incandescent lamps (25, 26). Because of the low precision of volatilization, this method results in loss of resolution. The new generation of TLC–LSIMS instruments overcomes this difficulty, and they can determine both mass spectra and mass chromatograms (27, 28). The theory, instrumentation, and practical applications of various TLC–MS systems have been reviewed extensively (29).

Although TLC–MS techniques offer a unique possibility for identifying solutes separated on TLC support, coeluted contaminants can influence the performance of TLC–MS sytems and can make identification questionable or more difficult. Use of TLC–MS–MS or other more complicated methods overcome this difficulty and provides more information regarding the structure of solutes than does the common TLC–MS technique (30). TLC–MS–MS and TLC–digital autoradiography have been used in metabolic research (31): Labeled metabolites of deramciclane, a new anxiolytic agent, were separated on silica gel plates. The positions of spots were determined with digital autoradiography, and metabolites were identified by TLC–MS–MS.

GC/MS also has been used to identify unknown compounds separated by TLC. This combined method was used to analyze hydroxy (32) and amino polycyclic aromatic hydrocarbons (33).

TLC–Raman Spectroscopy

Because TLC supports are weak Raman scatterers, Raman spectroscopy also can be used as a method of identification in TLC (34). Earlier it has been established that adsorption of organic molecules on (rough) metallic surfaces considerably enhances the sensitivity of the method (surface-enhanced resonance Raman spectroscopy; SERS; 35, 36). TLC/SERS is simple: TLC is performed in the traditional manner, and the developed plates are treated with silver sol and evaluated by a Raman equipment. Because of its high sensitivity, TLC–SERS detects compounds at nanogram levels (37). TLC–SERS has been used for a wide variety of solutes such as acridine orange, nitrogen-containing polyaromatic hydrocarbons (PAHs; 38), cetylpyridinium chloride (39), and aminotriphenylmethane dyes (40). Because the reproducibility of the preparation of silver sols is lower than that of any quantitative analyses performed by current TLC methods, TLC–SERS has been proposed as a means to facilitate identification of solutes and not for exact quantitative determination of solutes on the plate. Applications of both FT Raman and FT SERS with TLC plates have been demonstrated recently (41).

TLC–FTIR Spectrometry

Because FTIR spectrometry also has a high capacity of discrimination as do MS and Raman spectroscopy, the possibility of using FTIR spectrometry as a TLC detector has been studied many times (42, 43). TLC–FTIR can be performed by 2 entirely different experimental designs. The more elegant and simple method performs FTIR measurement of the solute directly (in situ) on the plate (44, 45). However, TLC supports are strong absorbers of infrared light and can interfere with the FTIR spectra of the solute molecule.

To avoid interference, the second method extracts the compounds from the plates and uses IR-transparent media for measurements. Obviously, this method is more time consuming, and the probability of introducing impurities into the sample is higher. As both methods have advantages and disadvantages, their application always depends on the characteristics of the TLC/solute system under investigation. Recently it was established that adenosine and related compounds also can be unambigously identified by TLC–in-situ FTIR (46).

TLC–Solid-Phase NMR

Although the information provided by NMR spectroscopy is very useful for identification of unknown solutes, this elegant method has not been used frequently in current TLC practice. The first attempt to use high-resolution magic-angle-spinning (HR MAS) solid-state NMR made use of octadecyl silica plates. Solutes were scrapped from the layer, slurried with D_2O, and analyzed by NMR (47). It was proven that the quantity of solutes spotted onto analytical TLC plates is sufficient for acquisition of NMR spectra. The special advantage of the method is its nondestructive character, allowing the solute to be investigated further by other spectroscopic methods.

TLC–AAS

Various AAS methods coupled with LC have been used to analyze metal complexes (48). A similar TLC method was developed to study the stability of zinc complexes (49): Zinc carboxylato complexes with 2 nicotinic acid ligands were separated on silica layers. Then the spots were scraped from the layer, and the concentration of zinc was determined by AAS. This hyphenated method allows determination of the stability of zinc complexes during separation and is suitable for detecting differences between the binding forces of the various ligands.

TLC–Square-Wave Stripping Voltammetry

This elegant method was developed for in situ detection of heavy-metal cations separated on TLC plates (50). A glassy carbon mercury film electrode was used as the working electrode for quantitative determination of Cd(II), Cu(II), Pb(II), and Zn(II). To prevent abrasion of the mercury film by the TLC support, the film was covered by a cellulose dialysis membrane. Method sensitivity was enhanced considerably by replacing the 3 mm electrode with a 10 μm electrode (51). This ultrasmall electrode allowed in situ detection of 1 ng Cd(II) and 4 ng Pb(II). The relationship between detector signal and amount of metal ions on the plate was significantly linear in the range of 20–500 ng, but spot-to-spot reproducibility of the method was relatively high.

TLC–HPLC

This coupling is not frequently used. However, an interesting application has been reported for analysis of complicated mixtures of PAHs (52): The effluent of PAH extract from marine sediments from a microbore columns was deposited on a linearly moving TLC plate. This combined method separated PAHs from each other, even those that coeluted during LC.

Conclusions

Hyphenated techniques play a considerable role in current TLC practice. These methods overcome some inherent problems associated with TLC analysis: ambiguous identification of solutes according to retention parameters and one or more color reactions and relatively low sensitivity and reproducibility of quantitative determination of some solutes without a chromophore in the molecule. However, hyphenated techniques do not increase the separation power of TLC; they only help detection and quantitative determination of solutes previously separated by traditional or instrumental TLC techniques without interfering with the separation.

Each hyphenated method was developed to facilitate analysis of a given solute. Thus, they cannot be used universally. Each method has advantages and disadvantages, and successful coupling to TLC depends on the expertise and consideration of the chromatographer. These methods are being developed continuously, and the number of studies dealing with hyphenated TLC techniques grows each year. Although use of hyphenated TLC methods is increasing, many other studies are needed to explore their use for a wide range of solutes, matrixes, and supports. It is highly probable that commercialization of various hyphenated TLC instruments will promote application of these powerful and efficient techniques.

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SPECIAL REPORTS

Contemporary Thin-Layer Chromatography in Pharmaceutical Quality Control

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The progress in layers, instrumentation, and the development of automated scanning densitometers have led to a remarkable improvement of the features of thin-layer chromatography (TLC) and especially its high performance version (HPTLC). Contemporary TLC or HPTLC combines the techniques' inherent advantages which include high sample throughput, simultaneous processing of standards and samples, flexibility, easy postchromatographic visualization techniques, and the ability to handle complex or crude samples with minimum sample cleanup—with improved selectivity, sensitivity, and accuracy. It is a very economical chromatographic technique that competes with and complements HPLC. Development and validation of procedures can be performed rapidly in full accordance with all current international guidelines.

Soon after the pioneering work of Stahl in the mid 1950s (1), thin-layer chromatography (TLC) found its way into pharmaceutical analysis. An early example of pharmacopoeial use was a general method featured in the British Pharmacopoeia (2). The introduction to that method refers to "... greatly increased emphasis placed on detection and control of impurities (from) manufacture or degradation ... made possible by the rapid development of TLC as a reliable means of detecting and assessing small quantities."

In the basic compendial mode, TLC is simple, rapid, robust, and inexpensive. Although sometimes not even mentioned in most of the latest textbooks, reviews, or editorials about chromatography, TLC is still widely used in pharmaceutical analytical laboratories throughout the world (3, 4) for identity testing. In its semiquantitative mode, TLC is used to detect and control impurities.

Even today, semiquantitative TLC procedures—where spots of diluted test solutions are visually matched against impurity spots in the chromatogram of undiluted test solution—are reliable enough to solve a number of analytical problems. The deaths of numerous children associated with improper use of glycerol or propylene glycol mixed with or containing ethylene glycols easily could have been avoided by quality control of the products by using TLC screening techniques, as proposed by Layloff et al. (5, 6).

However, most TLC procedures in pharmacopoeial monographs represent an obsolete standard of this chromatographic technique, leading to the misconception that TLC is merely a qualitative, or at best a semiquantitative, method with doubtful accuracy and sensitivity.

Meanwhile not only improvements of the compendial use of TLC have been suggested (7, 8). In addition, tremendous progress has been made in sorbent materials and layers, instrumentation, and automation. Development of sophisticated automated scanning densitomers has led to remarkable improvement of method features and reliability (9–16).

High performance TLC (HPTLC), characterized by use of layers composed of particles with smaller diameter and narrow particle size distributions, leads to greater separation efficiency, improved detection limits, and better quantitation. However, these developments seem to have been persistently ignored by pharmacopoeial authorities and by nearly all institutions involved in the education and training of pharmacists and analysts, especially in the United States.

A revised draft of the general monograph *Thin Layer Chromatography* of the European Pharmacopoeia was published recently. For the first time, it describes quantitative TLC by scanning densitometry and performance parameters for resolution and limit of detection (17, 18). Description of laboratory-prepared plates was deleted from the monograph, and commercially available plates are now refered to in the reagents section. A monograph, *Soya Lecithin*, drafted by the German Pharmacopoeial Commission includes an assay for phosphatidyl choline by quantitative TLC with scanning densitometry (19).

Nevertheless, quantitative TLC or HPTLC are not generally accepted analytical techniques in Europe. The rapid development of liquid chromatography (LC) in the mid-1970s has made LC the predominant mode of obtaining analytical information in pharmaceutical analysis. As a consequence, in an inquiry concerning

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the general revision for the 4th edition of the European Phamacopoeia, the following question was raised: "Should there be a general shift away from TLC methods to HPLC methods for the control of impurities?" (20).

There are scientific and economic reasons not to accept this uncritical general preference for one of 2 proven analytical methods. The weak points of LC must not be underestimated, and some of them can be compensated by TLC or HPTLC (21). Ironically, this weakness has become obvious in the latest draft monographs of the European Pharmacopoeia, like the one for medroxyprogesterone acetate, that have to admit that "unfortunately it was impossible to detect all related substances by HPLC, so a TLC test has also been introduced" (22).

Application of TLC and HPTLC

TLC and HPTLC procedures can be validated and performed rapidly and conveniently (23–27), in full accord with the latest guidelines of the International Conference on Harmonization (ICH; 28, 29). Performance data verify that, under optimized conditions, quantitative TLC or HPTLC produce results that are comparable with those of LC. Generally, selectivity, separation power, and sensitivity (limit of quantitation) are lower for TLC or HPTLC than for LC, but interestingly, we found that measurement uncertainty and precision data from validation experiments are comparable. It must be suspected that many impressive LC precision data are not generated correctly, and unqualified estimates of measurement uncertainty are reported. These result in misleading underestimation of the true variability of LC (30, 31).

Advantages of TLC and HPTLC include the following: (*1*) simplicity of handling and performance; (*2*) flexibility and short time to begin an analysis due to the broad variety of stationary phases, mobile phases, and operational aspects combined with the availability of sensitive and selective reagents for detection and/or visualization; (*3*) no obligation for elution; (*4*) the possibility to evaluate the whole chromatogram stored on the plate and, consequently, the ability to repeat detection or quantitation steps with different parameters; (*5*) high sample throughput and an increased reliability of results (in-system calibration) because of the ability to simultaneously, yet independently, develop several samples and reference standards on one plate; (*6*) robustness, allowing easy transfer and adoption; and (*7*) the ability to handle crude, complex, or dirty samples.

The cost-effectiveness of TLC and HPTLC make these techniques potential alternatives to more costly methods, especially when series of determinations like content uniformity tests have to be performed and automated or semiautomated LC systems are not available or practical from an economic or technical point of view; components with difficult detection characteristics in LC, capillary electrophoresis, or gas chromatography have to be analyzed; and sample composition requires laborious workup for other chromatographic techniques.

Economic Considerations

Pharmaceutical analytical laboratories work under enormous economic pressures. Investigation of laboratory or product failures call for additional non-value-adding tests. At the same time, personnel and operational costs must be reduced and sample throughput must be increased, both without any considerable consequence to the reliability of analytical results.

Cost reductions with use of TLC or HPTLC instead of LC can be significant, especially when many repetitive analyses have to be performed and the attainable separation and performance parameters are considered sufficient (32). The benchmarking in Table 1 compares 2 examples where HPTLC and LC procedures with similar performance and validation characteristics were used to analyze the same products.

Theophyllin tablets were assayed according USP XXIII (33) or our HPTLC procedure (27). In addition, performance data of an optimized LC procedure developed by a contract manufacturer are given. Content uniformity of methscopolamin tablets was tested ac-

Table 1. Benchmarking: HPTLC versus LC

			LC	HPLC	
Product	Test	Performance parameter	United States Pharmacopoeia	Optim	HPTLC
Theophyllin tablets	Assay	Working hours/assay	4.5	2.7	1.3
		Running costs, $/assay	≈ 40	≈ 14	≈ 6
Methscopolamin tablets, 2 mg	Content uniformity	Working hours/test	10.3	—	3.1
		Running costs, $/test	≈ 120	—	≈ 14

cording to USP XXII (34) or our HPTLC procedure (35). The running costs of LC exceed those of HPTLC by a factor of 6–8, and the personnel capacity used by a factor of 3. These estimations agree with reports from quality control departments of other pharmaceutical companies (36).

The economic advantage of TLC and HPTLC may be even more significant for purity tests of pharmacopoeial active ingredients. Most proposed LC procedures use universal gradient systems, running 60–90 min/injection.

Components with Difficult Detection Characteristics

Numerous classes of compounds exhibit little or no UV activity and therefore require special detection techniques in LC. On the other hand, TLC or HPTLC can use potentially hundreds of selective or universal reagents for convenient pre- or post-chromatographic derivatization (37, 38).

Quaternary ammonium antiseptics, such as benzalkonium chloride or cetalkonium chloride, are cationic surfactants with bactericidal activity that are used frequently in pharmaceutical preparations. Cetalkonium chloride conveniently can be assayed in semisolid preparations by HPTLC on silica gel, using *n*-butyl-alcohol–glacial acetic acid–water (66 + 17 + 17, v/v) as mobile phase and derivatization with hexaiodoplatinate reagent. Violet spots formed are scanned in absorbance mode at 600 nm, and quantitation is based on peak area.

The excellent recovery (99.4%, coefficient of variation [CV] = 3.2%, n = 8), demonstrates the method's accuracy. Calibration is linear from 60 to 140 μg/mL, a range covering more than the required working range. Repeatability is acceptable (relative standard deviation [RSD] = 4.6%, n = 8), given the poor precision of spectrophotometric assays and the presence of cetalkonium chloride in low concentration (0.01%), accompanied by 8.7% of choline salicylate, a second quaternary compound (39).

Changing to reversed-phase (RP) conditions with silanized silica gel as stationary phase and a mixture of methanol, 25% (m/v) aqueous sodium triacetate trihydrate solution, and acetone (65 + 35 + 20, v/v) allows even separation of chain homologues of various quaternary ammonium antiseptics, which can be visualized with a solution of KI_3 in methanol–water. Yellow-brown spots are scanned in the absorption mode at 400 nm. Peak areas of each homologue in commercial samples can be determined with good to fair precision (RSDs ranging from 2.1 to 5.0%, n = 4), confirming that the longer-chain homologues show a stronger germicidal activity (40).

Although LC is widely promoted for lipid analysis, several features make TLC and HPTLC still particularly useful for this application (41–43).

Defined phospholipids—either semiquantitative or produced by lecithin fractionation techniques—are used widely today as multifunctional excipients or active ingredients in pharmaceutical products. Analytical procedures to determine purity or assay must separate not only the different phospholipid classes but also the degradation products, such as lysolipids and phosphatidic acids. Because of the poor UV activity of these compounds, detection in LC requires UV measurements near 200 nm, thus excluding gradient elution or requiring use of light-scattering detection. TLC and HPTLC offer numerous visualization reagents and well-established analytical procedures.

1,2-Distearoylphosphatidyl glycerol (DSPG) can be assayed as bulk material or the pharmaceutical product can be determined in an ultrasound contrast medium by HPTLC on silica gel plates, using chloroform–methanol–glacial acetic acid–water (20 + 10 + 3 + 1.4, v/v) as developing solvent. After chromatography at 8°–9°C (5 cm, ca 15 min), the dried plates are dipped into copper(II) sulfate–phosphoric acid reagent. Brown-violet spots are scanned at 365 nm, and quantitative evaluation is based on peak areas. Under optimized conditions (automatic bandwise application, 3-point calibration), the procedure is well suited for determining DSPG content and purity.

For U.S. registration of DSPG, an alternative LC procedure using light-scattering detection was developed to avoid the predicted argumentation with the U.S. Food and Drug Administration (FDA) regarding the acceptability of TLC.

The LC procedure—intended for bulk assay only—uses a very sensitive, binary gradient. Each solvent system consists of 4 components, the diol column has to be maintained at 55°C, and the nonlinear response of the delicate light scattering detection requires an extensive replicate calibration routine. Even then, the validation data do not match the performace of the HPTLC procedure (Table 2), especially given the fact that the LC data correspond to bulk drug substance assay whereas the HPTLC data correspond to quantitation in the finished product containing only 10% DSPG in a complex matrix.

In addition, the LC procedure is not robust, failing to generate reliable calibrations when used in long-term studies. This observation is confirmed by other reports comparing LC and HPTLC in phospholipid analysis (44).

Table 2. Assay of DSPG content by LC and HPTLC

Validation parameter	LC[a]	HPTLC[b]
Recovery, %	101.3	100.8
Repeatability, RSD (n = 6), %	0.9	0.9
LOQ MSPG,[c] %	0.4, CV = 7%	0.8, CV = 1.4%

[a] Assay for bulk drug substance.

[b] Content of finished product containing 10% DSPG.

[c] Limit of quantiation, mono stearoly phosphatidyl glycerol.

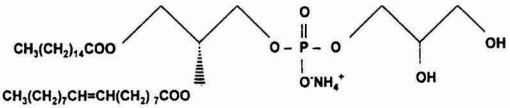

Figure 1. Structure of 1-palmitoyl-2-oleylphosphatidyl glycerol.

It is alarming that the anticipated negative reaction of FDA seems to have greater influence on selection of an analytical procedure than objective validation data have!

The power of HPTLC in phospholipid analysis is also demonstrated by the very similar purity tests of 1-palmitoyl-2-oleyl phosphatidyl glycerol (POPG, Figure 1), that allows detection and quantitation of unknown impurities to concentrations as low as of 0.05% (Figure 2).

Analysis of Complex or Crude Matrixes

One of the most outstanding features of TLC and HPTLC is the ability to handle crude or dirty samples. The presence of strongly absorbed impurities or even particles is of no concern, because plates are used only once, and the entire chromatogram can be evaluated. Compared with LC, TLC or HPTLC requires fewer, or even no, sample cleanup steps, saving both time and expense.

Sometimes interfering components may be removed conveniently with a first "cleaning" development step. In our purity test for fluphenazine decanoate in sesame oil, performed by TLC on silica gel, a first run with diethyl ether completely removes the sesame oil and preservatives. With diethyl ether, however, absolute absence of ether peroxides is mandatory to avoid analytical artifacts.

During development with ethyl acetate–methanol–diethylamine (80 + 10 + 5, v/v), fluphenazine decanoate, fluphenazine, the *S*-oxide, the aminoxide, and other unknown impurities are separated (45, 46). Under optimized conditions, limits of detection range from 0.1 to 0.3%, corresponding to 10–20 ng/band. No sample preparation is required; the oily liquid is diluted with acetone and automatically spotted to the plate. None of the tested LC procedures could separate and quantitate these impurities with wide-ranging polarities. Because these components originating from the sesame oil matrix change in nature and/or concentration from batch to batch, they heavily interfere with analysis of the natural excipient.

The ability of TLC and HPTLC to deal with complex matrixes and to allow different sequential detection is useful in analysis of topical preparations containing benzyl or methyl nicotinate and/or hydroxyethyl salicylate as rubefacients. These active ingredients are stable; the only degradation products are nicotinic acid and salicylic acid. HPTLC on silica gel with development at 6°C (6 cm, 20 min) using toluene–diethyl ether–glacial acetic acid (6 + 1 + 1, v/v) separates the active ingredients from matrix and impurities. Benzyl nicotinate and methyl nicotinate are scanned sequentially in the absorbance mode at 224 and 268 nm, respectively. Hydroxyethyl salicylate is detected in the fluorescence mode after excitation at 322 nm. Previous LC procedures required elaborate sample preparation to remove interfering matrix components such as polyacryl gel, glycerol monostearate, or complex emulsifying excipients. Whereas for HPTLC, the sample simply is diluted with isopropyl alcohol and spotted directly, leading to savings in time and expense. The validation data obtained with the product with the most complex composition prove the procedures' suitability (Table 3).

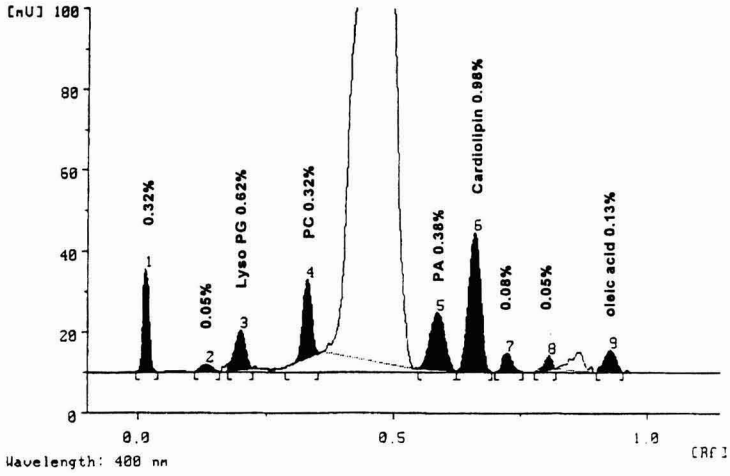

Figure 2. HPTLC analysis of 1-palmitoyl-2-oleylphosphatidyl glycerol (original scan, courtesy of S. Sadler, Genzyme Pharmaceuticals, UK).

Table 3. Validation data: active ingredients in topical antirheumatic ointment

Analyte	Accuracy, % (recovery, $n = 6$)	Precision, % (repeatability, $n = 6$)
Benzyl nicotinate	101.8	2.4
Methyl nicotinate	101.6	3.2
Hydroxyethyl salicylate	100.7	1.8

The ability of planar chromatography to cope even with exotic samples is demonstrated by the analysis of an NMR imaging agent based on supraparamagnetic Fe_3O_4 particles coated with poly(ethylene glycol) methyl ether phosphoric acid esters (Figure 3; 47).

To determine the degree of phosphoric ester coating and the composition of the ester, the imaging agent is centrifuged, the supernatant containing unbound ester is discharged, and the residue is dissolved in methanol with 2 drops ammonia added. This solution is spotted directly on HPTLC silica gel plates bandwise and developed with *n*-butyl–ethanol–25% aqueous ammonia (60 + 15 + 35, v/v) with chamber saturation. The coating is split off immediately, the iron oxide particles remain sorbed at the point of application, and the phosphoric acid esters are well separated from degradation products like diesters, polyethylene glycol mono- and diesters, or polyglycols. Derivatization with Dragendorff reagent results in orange-brown bands, which are scanned in the absorbance mode at 530 nm (Figure 4). In this application, the HPTLC plate has 3 functions: reaction enhancer for cleavage of coating, guard to retain iron oxide particles, and stationary phase (48).

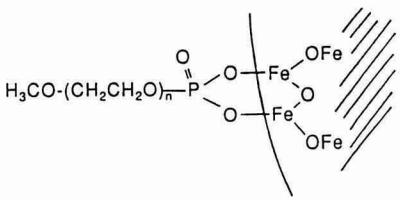

Figure 3. Structure of NMR imaging agent.

Plant Extracts or Phytopharmaceuticals

Identifications, assays, and purity test of plant extracts or phytopharmaceuticals are classical applications of TLC and HPTLC that take advantage of the features mentioned before: handling of very crude and dirty samples with ability to perform post- or prechromatographic derivatization. Approximately 1/3 of all TLC publications deal with analysis of plant ingredients; most are published in Chinese (16, 49).

Isolation and analysis of paclitaxel (taxol) from *Taxus baccata* or other species is complicated by the compound's low concentration and the presence of related compounds and closely related diterpenoids. A sensitive, simple, and reliable HPTLC procedure with prechromatographic derivatization that separates all components and allows quantitative determination was reported recently (50). Dried crude extracts from plant material are dissolved in a solution of dansyl chloride in acetone and applied to the plate, which is then exposed to triethylamine vapor to accomplish derivatization. Chromatographic separation uses cyclohexane–ethyl acetate–toluene–triethylamine–methanol (9 + 6 + 4 + 3 + 1, v/v) as developing solvent. Quanti-

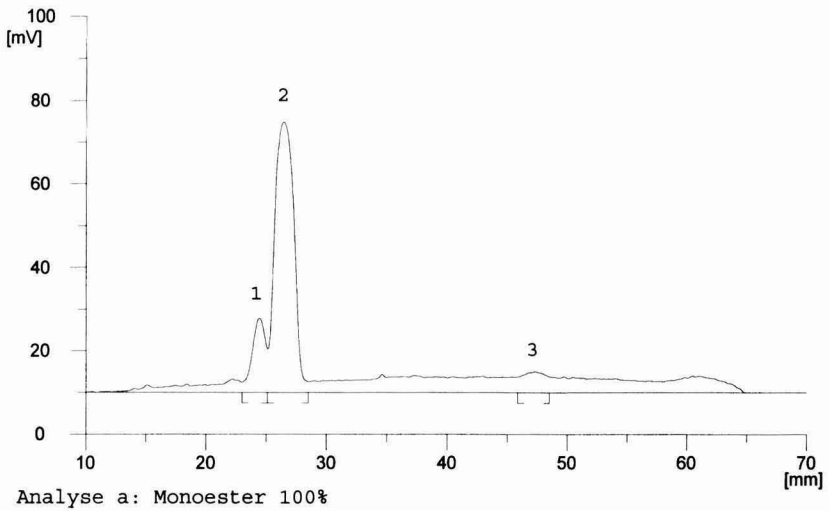

Figure 4. HPTLC analysis of NMR imaging agent. Peak 1: Phosphoric acid diester; peak 2: phosphoric acid monoester, peak 3: polyethylene glycol monomethyl ester.

tation is performed by scanning in the fluorescence mode after excitation at 254 nm and using a 440 nm filter. Calibration is linear from 20 to 200 ng, recoveries of the 4 main components are 95.7–104.3%, precision is acceptable (RSD is 2.2% for paclitaxel (taxol) and 2.3–4.4% for other components, $n = 8$), and fluorescence is stable for more than 3 h. The procedure is very useful in development and use of worldwide *Taxus* resources.

Future Perspectives

TLC and HPTLC are no longer the traditional, uncomplicated but less reliable methods. Today sophisticated automated instruments are available for individual steps like sample application, development, derivatization, scanning, and quantitation. A proposed, fully automatic TLC system (51) may not even be an advantage at all, because the flexible modular combination of individual steps is one of the main features of TLC and HPTLC.

The availability of a reliable, technically mature forced-flow system (overpressured layer chromatography) may positively influence the future of TLC and HPTLC: This technique eliminates the vapor phase, and the solvent phase is supplied by pump. Mobile-phase flow is constant but can be adjusted. It offers the possibility of longer development distances and thus better separation in less time (52).

The most promising development that might change the attitude of many analysts to TLC and HPTLC are the recently introduced video integration systems. Originally developed for documentation only, modern charge-coupled discharge video (CCD) cameras are now combined with sophisticated powerful software to image analyzing systems that can collect the information stored on a TLC or HPTLC plate in a very short time.

First reports comparing video integration and scanning densitometery showed the image-analyzing system to be less precise and accurate. Latest published results, however, indicate that, at least in the UV region, scanning densitometry and video integration produce analytical results that are equivalent in accuracy and precision (53–55). The decision to buy a smart up-to-date video integration system rather than a voluminous and possibly anachronistic scanner may be based not only on the decidedly lower price of the image-analyzing system but also on the higher acceptability of what is supposed to represent the latest technological standard.

It must be hoped that these developments will help TLC and HPTLC gain the reputation and acceptance they already deserves.

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AGRICULTURAL MATERIALS

Liquid Chromatographic Method for Determining Thiodicarb in Technical Products and Formulations: CIPAC Collaborative Study

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A liquid chromatographic method for determining thiodicarb in technical products and formulations was evaluated by 25 participants from 19 laboratories. Data from 19 laboratories were used in statistical analysis to characterize method performance. Two technical materials, a suspension concentrate, a wettable powder, and a water dispersable granule were analyzed. Thiodicarb was determined by reversed-phase liquid chromatography using a mobile phase of methanol and water. Chromatography was performed on a C_8 column with detection at 254 nm. Quantitation was achieved by using an internal standard and peak area ratios.

Thiodicarb, 3,7,9,13-tetramethyl-5,11-dioxa-2,8,14-trithio-4, 7, 9, 12-tetraazapentadeca-3, 12-diene-6,10-dione, is an insecticide/ovicide available as technical material, suspension concentrate, wettable powder, or water-dispersable granule. This insecticide/ovicide is used primarily against Lepidopterous insects.

In the collaborative study, samples of available technical materials and formulations were dissolved in dichloromethane. The internal standard in methanol was added, and analytes were separated by liquid chromatography (LC) on a reversed-phase column with a mobile phase of methanol–water (60 + 40, v/v) and UV detection at 254 nm. Peak area ratios were used for quantitation with an internal standard.

Submitted for publication November 17, 1997.

The recommendation was approved by the Committee on Pesticides and Disinfectant Formulations, and was adopted by the Official Methods Board of the Association. *See* "Official Methods Board Actions" (1997) *J. AOAC Int.* **80**, 126A, and "Official Methods Board Actions" (1997) *Inside Laboratory Management*, November issue.

Collaborative Study

The study was directed by Francis Gomez (Rhône-Poulenc Secteur Agro, Center de Recherche de La Dagroire, 14-20 Rue Pierre Baizet, 69009 Lyon, France) under the auspices of the French Pesticide Analytical Council. The study was presented at CIPAC Meeting 40, Beijing, China (CIPAC method document 3864/m and report document 3865/R).

Twenty-two laboratories agreed to participate in the collaborative study, and 19 laboratories returned analytical results. Three laboratories used a C_{18} vs a C_8 column as specified in the method. Data from all 19 laboratories were used to evaluate performance parameters after elimination of outliers following AOAC statistical protocols. Two technical materials, 1 suspension concentrate, 1 wettable powder, and 1 water-dispersable granule were sent to collaborators along with instructions and the analytical method. Each of the 5 samples was analyzed once on 2 different days.

997.14, Thiodicarb in Technical Products and Formulations, Liquid Chromatographic Method CIPAC–AOAC Method

First Action 1997

(Applicable to technical, suspension concentrate, wettable powder, and water dispersable granule formulations.)

Caution: See Appendix: Laboratory Safety for "Safe Handling of Organic Solvents"—dichloromethane and methanol.

Method Performance:

See Table **997.14** for method performance data.

A. Principle

Sample is dissolved in dichloromethane, the internal standard in methanol is added, and analysis performed

Table 997.14. Method performance for determination of thiodicarb in technical products and formulations by liquid chromatography

Product	No. of labs[a]	Mean, g/kg	s_r	s_R	r[b], g/kg	R[c], g/kg	RSD_r, %	RSD_R, %
Technical	19 (17)	950.0	9.25	9.25	25.90	25.90	0.97	0.97
	19 (18)	949.2	8.95	9.16	25.06	25.64	0.94	0.96
Suspension concentrate	19 (15)	342.3	3.94	6.43	11.04	17.99	1.15	1.88
Wettable powder	19 (17)	760.4	6.70	10.22	18.76	28.63	0.88	1.34
Water dispersable granule	19 (17)	791.8	5.54	7.30	15.22	20.44	0.70	0.92

[a] Number of labs participating (number retained in calculating method performance).
[b] $r = 2.8 \times s_r$.
[c] $R = 2.8 \times s_R$.

on a reversed-phase C_8 column with a water–methanol mobile phase. Thiodicarb is detected by absorbance at 254 nm and quantitated by peak area measurements based on an internal standard.

B. Apparatus

(a) *Liquid chromatograph.*—Equipped with a 10 μL sample loop injector (or autoinjector). Operating conditions: mobile phase flow rate 1mL/min (constant-flow pump); temperature, ambient.

(b) *Column.*—250 × 4.6 mm id, stainless steel packed with reversed-phase C_8 on 5 μm silica.

(c) *UV detector.*—Capable of measuring at 254 nm and set to give 80–90% full scale deflection for concentration of standard solution used for calibration.

(d) *Electronic integrator.*—Preferred for peak area measurements.

(e) *Sample filtration device.*—Glass syringe fitted with a membrane filtration unit compatible with organic solvents and filter (0.5 μm pore size).

(f) *Ultrasonic bath or mechanical shaker.*

C. Reagents

(a) *Calibration solution.*—Weigh in duplicate (to the nearest 0.1 mg) ca 0.08 g thiodicarb standard (of known purity, available from Rhône-Poulenc) into separate volumetric flasks (100 mL). To each, add 5 mL dichloromethane and 10.0 mL internal standard solution (solution I) from a pipette. After allowing solution to adjust to temperature, dilute to volume with methanol. Mix thoroughly (solutions C_1 and C_2). Filter through a 0.5 μm filter before LC.

(b) *Internal standard solution.*—Dissolve dimethyl phthalate (2 mL) in methanol (200 mL; solution I). Prepare a sufficient quantity of this solution for all samples to be analyzed and calibration solutions to be used in analysis. Filter.

(c) *Solvents.*—Water, methanol, and dichloromethane, LC grade.

(d) *LC mobile phase.*—Methanol–water (60 + 40, v/v).

(e) *Column flush.*—Methanol.

D. Preparation of Test Samples: Technical and Formulated Products

Into 100 mL volumetric flask, weigh (to nearest 0.1 mg) enough sample to contain ca 0.08 g thiodicarb. Add 5 mL dichloromethane and dissolve by shaking thoroughly. For suspension concentrate formulations, shake thoroughly or place flask in an ultrasonic bath for 2 min. Add by pipette internal standard solution (solution I; 10 mL) and dilute to volume with methanol after temperature equilibration. Mix thoroughly and filter through a 0.5 μm filter before analysis.

E. Determination

Before analysis, equilibrate column with mobile phase for 10 min. After sample analysis, flush column for 10 min with methanol and equilibrate column again with mobile phase before additional sample analysis.

Inject first calibration solution (C_1), first sample solution twice, and then the second calibration solution, etc. (C_1, S_1, S_1, C_2, flush, equilibrate, C_1, S_2, S_2, C_2). Determine peak areas for sample and standard injections.

F. Calculations

Calculate response factor, R, for each injection as follows:

$$R = \text{area thiodicarb/area internal standard}$$

Calculate thiodicarb in sample as follows:

$$\text{Thiodicarb, \%} = R/R^1 \times W^1/W \times P$$

where R and R^1 = average response factors for sample and standard solution injections, respectively; W and W^1 = weights (mg) of sample and standard, respectively; and P = purity (%) of standard.

Ref.: *J. AOAC Int.* **81**, 341 (1998).

Results

Twenty-two laboratories agreed to participate in the study and received samples, the method of analysis, and instructions. Three laboratories withdrew: one had a high work load and another could not recover samples from customs. Results of analysis from 19 laboratories were received for statistical evaluation.

Three laboratories out of the 19 participants returning results used a C_{18} column instead of a C_8 column as indicated in the proposed analytical method. These laboratories produced good results and were retained for determination of method performance.

The most recent AOAC statistical protocols were followed to analyze all the data and remove Cochran and Grubbs outliers. Results of the statistical evaluation can be found in Table **997.14**. A total of 11 outliers were identified and eliminated for determination of the statistical performance parameters for the Thiodicarb method.

Collaborators' Comments

One laboratory encountered difficulties in dissolving the suspension concentrate sample: membranelike floating particles could be observed.

Another laboratory suggested rejecting the result for the suspension concentrate sample on day one because of an identified preparation error.

A third laboratory indicated that most peaks showed an inflection point.

To avoid eluting thiodicarb after the flush was started, a laboratory used a flow rate of 1.3 mL/min.

It was suggested that the suspension concentrate formulation be dissolved by adding a few drops of water before adding dichloromethane to prevent precipitation and lumping.

An improvement to sample preparation was recommended by a laboratory suggesting a volume of methanol be added to each sample followed by treatment in an ultrasonic bath and then vigorous shaking before filtration.

Recommendation

Final results for the collaborative study for determining thiodicarb in technical products and formulations are well within acceptable limits. Thus, the LC method tested is recommended for adoption as an AOAC first action method.

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AGRICULTURAL MATERIALS

Quantitation of Imidacloprid in Liquid and Solid Formulations by Reversed-Phase Liquid Chromatography: Collaborative Study

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Collaborators: Richard Ambrose, Lajos Benke, Ralph Denning, B. de Ryckel, Charles Focht, Bura Laslo, Lalini Manuweera, C.L. Ritland, A. Richard Smilo, Steven Stroh, K.C. Tam, H. Tengler, Gu Yi Xing

A liquid chromatographic (LC) method was developed for quantitation of the synthetic insecticide imidacloprid in liquid and solid formulations. Samples are dissolved or extracted in solvent and analyzed by reversed-phase LC with propiophenone as internal standard. Fourteen laboratories in 7 countries participated in the collaborative study of the method. Each collaborator was provided with reference standard, internal standard, and matched-pair samples of imidacloprid technical, flowable, wettable powder, fertilizer, and granular formulations. Collaborators were instructed to use peak area measurements for quantitation. The reversed-phase LC method for determination of imidacloprid in liquid and solid formulations has been adopted first action by AOAC INTERNATIONAL.

Imidacloprid, 1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidimine, is available in 75% wettable powder (WP), 20% flowable, fertilizer (0.1–0.5%), and several granular formulations (0.1–1%). Imidacloprid is a systemic insecticide with low mammalian toxicity. It is primarily effective for control of sucking insects such as grubs and whiteflies. It is also very effective on grubs and termites. It is used on a variety of vegetables, field crops, fruit crops, and ornamentals. It is effective on turf for the entire summer growing season. It can be used as a seed, soil, or foliar application (1).

Collaborative Study

Fourteen collaborators in 7 countries volunteered to take part in the study. The study was designed according to the suggestions of Youden and Steiner (2). Each collaborator received a copy of the analytical method, matched pairs of each formulation, reference standard, internal standard, practice sample, and material safety data sheet. Collaborators were instructed to store test samples and the standard under refrigeration until the beginning of the study and to allow the materials to equilibrate to room temperature before opening them. Peak areas were specified for quantitation but peak heights were allowed if an integrator was unavailable.

Submitted for publication June 13, 1997.
The recommendation was approved by the Methods Committee on Pesticides and Disinfectant Formulations, and was adopted by the Official Methods Board of the Association. *See* "Official Methods Board Actions" (1997) *J. AOAC Int.* **80**, 84A, and "Official Methods Board Actions" (1997) *Inside Laboratory Management*, August issue.

997.12, Proposed Method for Imidacloprid in Pesticide Formulations

First Action 1997

(Applicable to technical, wettable powder [WP], flowable [F], fertilizer, and granular formulations.)

Caution: See Appendix: Laboratory Safety for "Safe Handling of Organic Solvents" and "Safe Handling of Special Chemical Hazards"—pesticides, methanol, acetonitrile, and methylene chloride. Imidacloprid is an inhalation, ingestion, and absorption hazard; use protective gloves. *See* material safety data sheets, or equivalent, for each reagent. Dispose of waste solvents and pesticide solutions in an appropriate manner compatible with applicable environmental rules and regulations.

Mammalian toxicity values (mg/kg) for technical-grade imidacloprid (LD_{50}) are male rat, 424 (oral) and >5000 (dermal); female rat, 450–475 (oral) and >5000 (dermal).

Method Performance:
See Table **997.12** for method performance data.

A. Principle

Imidacloprid is determined by liquid chromatography (LC). Peak areas of sample and standard are compared by using propiophenone as internal standard.

Table 997.12. Method performance for LC determination of imidacloprid in pesticide formulations

Statistic	Technical	75% WP	20% Flowable	Fertilizer	Granules
Mean	97.73	74.96	21.44	0.51	1.05
s_r	0.49	0.34	0.09	0.01	0.03
r	1.38	0.95	0.26	0.02	0.08
s_R	1.12	1.25	0.48	0.03	0.03
R	3.14	3.49	1.35	0.08	0.10
RSD_r, %	0.50	0.45	0.43	1.50	2.85
RSD_R, %	1.15	1.66	2.25	5.71	3.28
No. of laboratories	12	12	10	11	12

The method is applicable to formulated products containing imidacloprid as the only active ingredient.

B. Apparatus

(a) *Liquid chromatograph.*—Able to generate >7 MPa (>1000 psi). Equipped with a spectrophotometer to measure absorbance at 252 nm and peak area integrator. Operating conditions: ambient column temperature; flow, ca 1.2 mL/min (ca 1800 psi); recorder speed, 0.5 cm/min; recorder range 10 mV; injection volume, ca 1 μL for fertilizer determinations and ca 10 μL for all others; absorbance range, 1.0 absorbance unit full scale. Retention times: imidacloprid, ca 2.1 min; propiophenone internal standard, ca 4.1 min. Pump LC mobile phase through column for at least 15 min and until system is equilibrated (flat baseline).

(b) *Chromatographic column.*—250 × 4.6 mm id packed with 5 μm octyldecylsilane-bonded silica gel.

(c) *Filters.*—0.45 μm porosity, solvent compatible.

(d) *Mechanical shaker.*

(e) *Sampler.*—Riffle type.

(f) *Bath.*—Ultrasonic.

C. Reagents

(a) *Acetonitrile.*—LC grade or distilled in glass.

(b) *Water.*—LC grade or distilled in glass.

(c) *Methanol.*—LC grade or distilled in glass.

(d) *LC mobile phase.*—Acetonitrile–water (60 + 40), thoroughly degassed.

(e) *Internal standard solution.*—2.5% (w/v) propiophenone (Aldrich Cat. No. P5,160-5 or equivalent) in methanol.

(f) *Imidacloprid reference standard.*—From Bayer Corp. (Agriculture Division, PO Box 4913, Hawthorne Rd, Kansas City, MO 64120-0013); store under refrigeration when not being used. Reequilibrate to room temperature before opening.

D. Preparation of Standard Solutions

Because of the wide variety of matrix types, imidacloprid standard solution is prepared differently depending on the formulation being analyzed.

(a) *Technical, WP, and flowable formulations.*—Accurately weigh to ±0.0001 g ca 0.2 g imidacloprid reference standard into a 4 oz bottle. Pipet 5.0 mL internal standard solution into the bottle. Add 95 (±10) mL acetonitrile to the bottle and cap it with a Polyseal lid. Sonicate contents in an ultrasonic bath for 1 min and then mix thoroughly. Transfer 100 (±20) μL of this stock solution into a 2 mL autoinjection vial. Add ca 1–1.5 mL acetonitrile to the vial, cap, and mix thoroughly for determination. Label as standard solution S1.

(b) *Fertilizer formulations.*—Accurately weigh to ±0.0001 g ca 0.2 g imidacloprid reference standard into an 8 oz bottle. Pipet 5.0 mL internal standard solution into the bottle. Add 95 (±10) mL methanol to the bottle and cap it with a Polyseal lid. Sonicate contents in an ultrasonic bath for 1 min and then mix thoroughly. Transfer 1–1.5 mL of this solution into a 2 mL autoinjection vial and cap. Label as standard solution S2.

(c) *Granular formulations.*—Accurately weigh to ±0.0001 g ca 0.2 g imidacloprid reference standard into an 8 oz bottle. Pipet 5.0 mL internal standard solution into the bottle. Add 150 (±10) mL acetonitrile to the bottle and cap it with a Polyseal lid. Sonicate contents in an ultrasonic bath for 1 min and then mix thoroughly. Transfer 100 (±20) μL of this stock solution into a 2 mL autoinjection vial. Add ca 1–1.5 mL acetonitrile to the vial, cap, and mix thoroughly for determination. Label as standard solution S3.

E. Preparation of Samples

(a) *Technical, WP, and flowable materials.*—Accurately weigh an amount of sample to ±0.0001 g that represents ca 0.2 g technical, ca 0.3 g 75% WP formulation, or ca 1.0 g flowable formulation into a 4 oz bottle. *Note:* The technical and 75% WP formulation do not require mixing or grinding. The flowable formulation must be thoroughly mixed with a spatula, spoon, or similar device. It is especially important to dislodge any "caked" material from the bottom or sides of the container, redisperse it, and then vigorously shake the material for 1 min before sampling.

Add 10 mL LC grade water into each bottle containing flowable formulation and swirl contents to mix. Sonicate contents for 1 min in an ultrasonic bath. Do not add water to other formulations.

Pipet 5.0 mL internal standard solution into each bottle. Add 95 (±10) mL acetonitrile to the bottle and cap it with a Polyseal lid. Sonicate contents in an ultrasonic bath for 1 min and then mix thoroughly.

Filter each sample solution through separate 0.45 μm porosity filters. Transfer 100 (±20) μL of each filtrate into separate 2 mL autoinjection vials. Add about 1–1.5 mL acetonitrile to each vial, cap, and mix thoroughly for determination.

Inject solutions onto chromatograph according to procedure *F. Determination*, using standard S1.

(b) *Fertilizer.*—Pour entire sample across the center of a riffle-type sampler, collecting riffled portions in metal trays. Select one portion to continue riffling until a riffled portion of sample is 35–45 g. Weigh the entire portion to ±0.01 g into an 8 oz bottle. Take care to transfer all dust weighed into the 8 oz bottle.

Pipet 5.0 mL internal standard solution into the bottle. Add 95 (±10) mL methanol to each bottle and cap it with a Polyseal lid. Shake bottles horizontally on a mechanical shaker for 60 min. Allow solutions to settle after shaking. Filter a portion of each sample solution through separate 0.45 μm porosity filters into separate 2 mL autoinjection vials for determination.

Inject solutions onto the chromatograph according to the procedure *F. Determination,* using standard S2.

(c) *Granular formulations.*—Pour the entire sample across center of a riffle-type sampler, collecting riffled portions in metal trays. Select one portion to continue riffling until a riffled portion of sample is 35–45 g. Weigh entire portion to ±0.01 g into an 8 oz bottle. Take care to transfer all dust weighed into the 8 oz bottle.

Pipet 5.0 mL internal standard solution into each bottle. Add 150 (±10) mL acetonitrile to each bottle and cap it with a Polyseal lid. Shake bottles on a mechanical shaker for 60 min. Allow solutions to settle after shaking.

Filter each sample solution through separate 0.45 μm porosity filters, collecting 100 (±20) μL into separate 2 mL autoinjection vials. Add ca 1–1.5 mL acetonitrile to each vial, cap, and mix thoroughly for determination.

Inject solutions onto the chromatograph according to procedure *F. Determination,* using standard S3.

F. Determination

Adjust operating parameters so that elution times of imidacloprid and propiophenone peaks are 1.9–2.5 min and 4.0–4.4 min, respectively. A typical chromatogram is shown in Figure **997.12**. Make repetitive injections of the appropriate standard solution and calculate response ratios by dividing imidacloprid peak areas by that of internal standard peak areas. Response ratios for standard injections (R) must agree within ±1% for 2 consecutive injections before analysis is continued. Inject duplicate amounts of each sample solution (no more than 3 samples; i.e., 6 injections) between bracketing standard injections. Calculate the response ratios of sample injections (M) by dividing internal standard peak areas by imidacloprid peak areas. Response ratios of sample injections must agree within ±1.0% (±0.5% of their average). If not, repeat the determination starting with standard injections. Reinject the appropriate standard solution. Average response ratios of standard injections immediately preceding and following the sample injections. These must agree within ±1.0% (±0.5% of their average). Repeat any portion of the determination that does not meet this criterion.

G. Calculation

Calculate imidacloprid concentration (%) as follows:

$$\text{Imidacloprid, \%} = \frac{W_S \times P \times M_{av}}{W \times R_{av}}$$

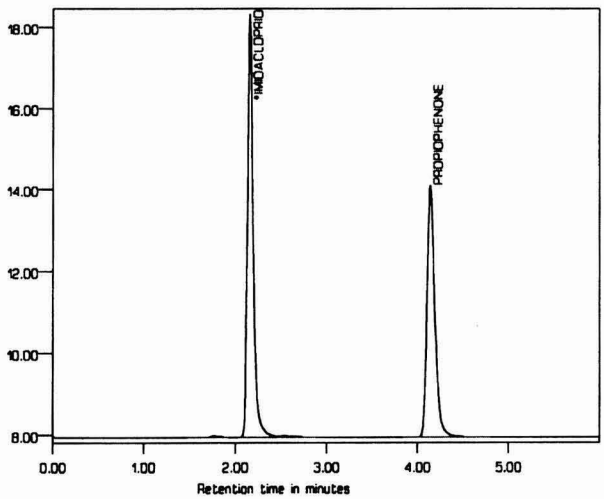

Figure 997.12. Typical chromatogram of imidacloprid formulation.

where W_S = weight of reference standard (g), P = percentage purity of reference standard, S = imidacloprid peak area in standard, I = internal standard (propiophenone) peak area of standard injection, W = weight of sample (g), A = imidacloprid peak area in sample, B = internal standard peak area in sample injection, standard response ratio (R) = S/I, sample response ratio (M_{av}) = A/B.
Ref.: *J. AOAC Int.* **81**, 343 (1998).

Results and Discussion

All LC analytical columns used in the study were octadecylsilane, but 12 different columns were used (Table 1).

Twelve of 14 collaborators successfully analyzed the complete set of test samples (Table 2). One collaborator had excessively large peak areas representing a missed dilution step. Another collaborator had an improperly prepared internal standard solution. Data from these 2 collaborators were rejected prior to statistical analysis.

Table 1. Columns used in study

Packing (size, μm)	Dimensions, mm
Zorbax Rx-C_{18} (5)	250 × 4.6
Spherex C_{18} (5)	250 × 4.6
CLC-ODS (5)	150 × 6.0
HP Hypersil ODS (5)	250 × 4
Chromsep SS C_{18} (5)	150 × 4.6
Zorbax ODS (5)	250 × 4.6
Alltech Altima C_{18} (5)	250 × 4.6
Zorbax ODS (10)	250 × 4.6
Ultramex C_{18} (5)	250 × 4.6
Ultramex C_{18} (3)	250 × 4.6
Alltech Hypersil ODS (5)	250 × 4.6
LiChroCart Supersphere 60 RP	250 × 4.6

AOAC INTERNATIONAL's statistical program AOACYMP was used for all calculations and for outlier search by Cochran and Grubbs' single value and Grubbs' double-value tests. Two pairs of the flowable formulation results and one pair of fertilizer results were eliminated from the data set on the basis of Cochran's test. All 12 pairs of results were used for all other formulations.

In granular and fertilizer formulations, imidacloprid is coated on the outside of the granule or fertilizer beads. To obtain a uniform sample, these formulations must pass through a riffler, a sampling device made of a set of metal fins designed so the sample is split in half each time it is poured across the apparatus. The 2 portions of the sample are collected in trays below. Splitting is repeated until the portion collected in the tray is within the sample weights specified in the method. The entire sample supplied is then used for analysis.

Collaborators' Comments

Most laboratories did not report problems with the method. Some laboratories changed the injection volume to avoid exceeding detector limits.

One laboratory with poor precision on the technical sample suggested using acetonitrile–water (50 + 50) for preparing the dilution in the injection vial. They thought the peak shape they obtained would be improved by reducing the level of organics in the injection vials. They considered a 10 μL injection volume to be high. This collaborator also suggested that the loss of precision on the technical sample may have been caused by a too short sonication time during sample preparation.

Conclusion

The results show this method is applicable to liquid and solid formulations of imidacloprid. The robustness

Table 2. Collaborative results of LC determination of imidacloprid (%) in formulations as matched-pair samples

	Technical		75% WP		20% Flowable		Fertilizer		Granules	
Collaborator	A	B	A	B	A	B	A	B	A	B
1	96.62	98.04	74.62	73.68	21.20	21.12	0.48	0.48	1.06	1.06
2	98.18	97.14	74.10	74.64	21.23	21.36	0.53	0.52	1.02	1.00
3	99.26	99.06	75.68	75.86	21.35	21.37	0.51	0.50	1.07	1.13
4	96.91	96.71	73.36	73.08	21.08	21.33	0.69[a]	0.53[a]	0.95	1.06
5	98.44	98.06	76.15	76.73	21.98[a]	21.39[a]	0.55	0.56	1.10	1.05
6	98.58	97.82	72.89	72.45	20.93	20.85	0.53	0.54	1.02	1.05
7	98.06	98.65	76.24	76.11	21.73	21.63	0.52	0.54	1.05	1.07
8	98.39	97.59	75.42	75.48	22.04	21.85	0.53	0.53	1.05	1.08
9	99.18	98.62	75.49	74.82	20.91	20.73	0.49	0.51	1.05	1.05
10	96.20	96.94	75.26	75.93	21.68	21.68	0.45	0.45	1.04	1.03
11	98.16	98.16	74.26	74.32	22.37	22.33	0.51	0.51	1.04	1.04
12	95.26	95.46	76.26	76.28	23.39[a]	22.23[a]	0.51	0.52	1.02	1.04

[a] Pair eliminated on the basis of Cochran's test.

of the method is exemplified by its successful application on 12 different columns.

Recommendation

On the basis of the results of this study, it is recommended that the LC method for determining imidacloprid in liquid and solid formulations be adopted first action.

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Gu Yi Xing, Shanghai Import & Export Commodity Inspection Bureau of the Peoples Republic of China, Shanghai, China

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AGRICULTURAL MATERIALS

Reversed-Phase Ion-Pair Liquid Chromatographic Determination of Chlorophacinone and Diphacinone in Steam-Rolled Oat Baits and Steam-Rolled Oat/Wax Baits

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A reversed-phase ion-pair liquid chromatographic (LC) method was developed for analysis of steam-rolled oat (SRO) baits fortified with either chlorophacinone or diphacinone. Baits were prepared with and without paraffin wax. Chlorophacinone or diphacinone was extracted from wax-free SRO baits with 5 mM tetrabutylammonium phosphate methanolic ion-pairing solution. Wax baits were initially extracted with petroleum ether and then cleaned up by liquid extraction into methanolic ion-pairing solution containing 20% water. SRO extracts were analyzed with reversed-phase ion-pair LC. Chlorophacinone and diphacinone were quantified by UV absorption at 325 nm. Recoveries from SRO fortified with chlorophacinone at 25 and 150 μg/g were 90.7 and 90.8%, respectively, whereas for diphacinone at the same levels, recoveries were 93.5 and 92.3%, respectively. Recoveries from wax baits fortified at 25 and 75 μg/g chlorophacinone were 98.5 and 100%, respectively, whereas for diphacinone at the same levels, recoveries were 93.6 and 98.0%, respectively. Method limits of detection for chlorophacinone and diphacinone in SRO baits were estimated to be 1.0 and 0.76 μg/g, respectively. Method limits of detection for chlorophacinone and diphacinone in wax baits were estimated to be 4.2 and 2.8 μg/g, respectively.

Diphacinone (2-(diphenylacetyl)-1H-indene-1,3(2H)-dione) and chlorophacinone (2-[(4-chlorophenyl)phenylacetyl]-1H-indene-1,3(2H)-dione) are registered anticoagulant rodenticides commonly used for controlling rats at dosage levels below those required by most other anticoagulant rodenticides. These anticoagulants also are effective in control of rangeland rodents such as Valley pocket gophers (*Thomomys bottae*), Belding ground squirrels (*Spermophilus beldingi*), and California ground squirrels (*Spermophilus beecheyi*). Pocket gophers and ground squirrels are vectors for diseases such as bubonic plague. These rangeland rodents can also reduce vegetation by 20 to 30%, which results in less plant material for livestock grazing. Additionally, the combination of grazing by pocket gophers, ground squirrels, and livestock can lead to severe soil erosion. Damage to earthen irrigation ditches and dams has been observed in areas where pocket gopher and ground squirrel populations have become excessive (1, 2). Control methods for ground squirrels and pocket gophers include exclusion, shooting, trapping, flooding, and use of acute toxicants including acute anticoagulants and fumigants (3). Steam-rolled oat (SRO) baits fortified at 50 and 100 μg/g chlorophacinone or diphacinone are used in California grasslands to control rodent populations. Wax baits fortified at 50 μg/g chlorophacinone or diphacinone are used in wetter regions of California to control rodent populations. Chlorophacinone- and diphacinone-fortified baits are formulated by small independent companies with limited quality control resources. To assist with registration of these formulations for protection of agriculture and public health, we developed practical methodology to verify the concentration of the active ingredients in these baits.

Several methods have been developed for analysis of indanediones in baits, formulations, and tissues. Each of these methods has some advantages and disadvantages. Gas chromatographic methods with derivatization (4) are sensitive and selective but suffer from low recoveries and lengthy preparation time. Spectrophotometric methods (5) have been used for baits and formulations, but they are not selective when multiresidue samples are being assayed. Thin-layer chromatographic (6–8) methods are not suited for determination of low levels of residues in complex matrixes or for accurate quantitation. Reversed-phase high performance liquid chromatography (LC) methods (9–13) provide the required sensitivity but often produce poor chromatographic resolution. Reversed-phase ion-pair LC (14–17) has adequate sensitivity and selectivity, but column deterioration is often a problem.

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Reversed-phase ion-pair LC was evaluated as the most appropriate method of analysis for our purposes because of the potentially good chromatographic resolution; column deterioration can be controlled reasonably well with column washing if it is done regularly (18). This method was simple and rapid. This method was validated for ground SRO containing 25 to 150 μg/g chlorophacinone or diphacinone and for wax baits containing 25 to 75 μg/g chlorophacinone or diphacinone.

Experimental

Apparatus

The LC system consisted of a Hewlett-Packard 1090 liquid chromatograph (Palo Alto, CA) operated at 35°C. A Hewlett-Packard 1050 variable-wavelength detector at 325 nm was used to detect chlorophacinone and diphacinone. The analytical wavelength of 325 nm was chosen over the more sensitive wavelength of 285 nm because the occurrence of a late-eluting peak is minimized when 325 nm is used as the analytical wavelength. A pneumatically controlled injector valve automatically injected 25 μL portions into the chromatograph. Analytes were separated on a 25 × 0.46 cm id stainless steel analytical column packed with 5 μm Keystone ODS/H (Bellefonte, PA) with a flow rate of 1.0 mL/min. To prolong column lifetime, a 1.5 × 0.46 cm id Keystone ODS/H guard column was used. The mobile phase was prepared by mixing aqueous and methanolic solutions of 5 mM tetrabutylammonium dihydrogen phosphate (20 + 80, v/v) and adjusting pH to 7.5 with 4N phosphoric acid. The mobile phase was degassed by sparging with helium. At the end of each set of analyses, the column was washed with methanol–water (1 + 1, v/v) for 40 min.

Operating conditions were adjusted occasionally to maintain optimum response and reproducibility. With these conditions, retention times of diphacinone and chlorophacinone were ca 4.5 and 6.5 min (Figure 1).

Reagents

Petroleum ether, ethyl acetate, and methanol were LC grade (Fischer Scientific, Denver, CO). Deionized water was purified with a Milli-Q water purification system (Millipore, Bedford, MA). Concentrated phosphoric acid (Fischer Scientific) was used to make 4N phosphoric acid in water.

Tetrabutylammonium dihydrogen phosphate (97%) from Aldrich (Milwaukee, WI) was used to prepare the 5 mM solution in methanol. A commercially prepared tetrabutylammonium dihydrogen phosphate ion-pairing reagent with buffer (potassium dihydrogen phosphate) was purchased from Alltech, Inc. (Deerfield, IL) and used to make the 5 mM solution in water.

Indanedione Standards

Chlorophacinone (98.9%) was obtained from LiphaTech (Milwaukee, WI), and diphacinone (99.3%) was obtained from Hacco, Inc. (Madison, WI). All concentrated and fortification standard solutions were prepared as separate solutions of chlorophacinone or diphacinone and not combined standards.

(a) *Concentrated stock standards and fortification standards (1000 μg/mL chlorophacinone or diphacinone).*—Prepared by first drying the technical-grade compounds for 4 h at 110°C and then dissolving 10.000 mg analyte in ethyl acetate in a 10 mL volumetric flask and diluting to volume with ethyl acetate.

(b) *Fortification standards (10 000 μg/mL chlorophacinone or diphacinone).*—Prepared by dissolving previously dried 100.00 mg diphacinone or chlorophacinone in ethyl acetate in a 10 mL volumetric flask and diluting to volume with ethyl acetate.

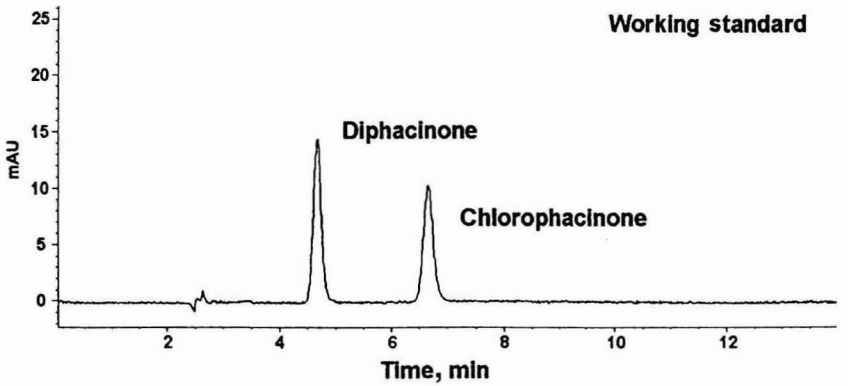

Figure 1. Chromatograms of a 1.0 μg/mL chlorophacinone and 1.0 μg/mL diphacinone working standard with ultraviolet detection at 325 nm.

(c) *Working standards ranging in concentration from 0.8 to 22.0 μg/mL.*—Prepared by diluting stock solutions with mobile phase. All standard solutions were stored in a refrigerator at 5°C.

Fortification of Control SRO Baits

Control baits consisted of SROs, Alcolec S as a binder, and Dupont Oil Blue A as a marker dye. Control baits were ground to a fine powder with an electric coffee mill (Krups, Type 203B) and stored in a sealed container. The method was validated at 2 levels of chlorophacinone and diphacinone: 25 and 150 μg/g. Each 1.00–1.10 g portion of ground SRO bait was fortified with one analyte by adding 25.0 μL of the 1000 μg/mL or 15.0 μL of the 10 000 μg/mL fortification standard solution in ethyl acetate to produce the appropriate fortification level. Tubes containing fortified SRO controls were then placed under a stream of nitrogen to evaporate the ethyl acetate from the fortification standard.

Fortification of Control Wax Baits

Control wax baits consisted of SROs, paraffin wax, Alcolec S as a binder, and Dupont Oil Blue A as a marker dye. Control baits were ground with a hand-powered grinding mill (Fischer Scientific) into pieces of wax and oats no larger than a quarter of an inch in diameter. This was then ground into a fine powder with an electric coffee mill and stored in a sealed container. The method was validated at 2 levels of chlorophacinone and diphacinone: 25 and 75 μg/g. Each 1.00–1.10 g portion of ground wax bait was fortified with either chlorophacinone or diphacinone by adding 25.0 or 75.0 μL of the 1000 μg/mL standard solution in ethyl acetate to produce the appropriate fortification level. Tubes containing fortified controls were then placed in a warm water bath at 70°C to melt the wax and encapsulate the analytes as in the actual baits, as well as to evaporate the ethyl acetate from the fortification standard.

Sample Extraction

(a) *Extraction of SRO baits.*—Ground SRO samples were weighed accurately in 1.00 g portions into a 50 mL screw-cap polypropylene tube. Then 10.0 mL methanolic ion-pairing solution was pipetted into the sample tube. The tube was shaken on a Vortex mixer for 10 s and then shaken horizontally with a mechanical shaker (Eberbach Corp., Ann Arbor, MI) at high speed for 15 min. Sample tubes were then sonicated for 3 consecutive 15 min periods, with the tubes shaken by hand for a few seconds between each period. Sample tubes were centrifuged at ca 2500 rpm for 5 min. A portion of the extract was filtered with a 0.45 μm Teflon syringe filter into a 2 mL sample vial, the vial was capped, and the sample was analyzed by LC.

(b) *Extraction of wax baits.*—Ground wax samples were weighed accurately in 1.00 g portions into a 50 mL screw-cap polypropylene tube. Then 20 mL petroleum ether was poured into the sample tube. The tube was shaken on a Vortex mixer for 10 s and then shaken horizontally with a mechanical shaker (Eberbach Corp.) at high speed for 15 min. Sample tubes were then sonicated in a beaker for 3 consecutive 15 min periods, with the tubes shaken by hand for a few seconds between each period. Then 20 mL methanolic ion-pairing solution with 20% water was pipetted into the sample tube. The tube was shaken on a Vortex mixer, shaken horizontally, and centrifuged as was done with the wax-free SRO baits. The petroleum ether layer (top layer) was removed from the tube, and the methanolic layer was transferred to a 25 mL volumetric flask. The sample tube was washed with two 1.5 mL portions of the methanolic solution, and the washes were transferred to the 25 mL volumetric flask, diluted to volume with the methanolic solution, and mixed well. The methanolic extract was filtered with a 0.45 μm Teflon syringe filter into a 2 mL sample vial. The vial was capped, and the sample analyzed by LC.

Results and Discussion

Response Linearity

Two sets of 5 calibration standard solutions were prepared, ranging in concentration from 0.8 to 22 μg/mL. Each standard solution was injected 2 times, and a linear regression was performed on the data set. The regression statistics are shown in Table 1.

A linear relationship existed between analyte chromatographic peak response and analyte concentration, and the response was directly proportional to concentration over the range of interest. Single-point calibrations were valid over the range of standard solution concentrations.

Extraction

Diphacinone and chlorophacinone residues were extracted from ground samples with methanolic ion-pairing solution. For wax baits, petroleum ether was used to dissolve the wax to allow encapsulated analytes to be extracted with the methanolic ion-pairing:water solution.

Table 1. Regression statistics

Compound	r^2	Slope	y intercept
Peak response vs concentration			
Diphacinone	0.9997	71.99	0.494
Chlorophacinone	0.9996	63.95	0.155
Log (peak response) vs log (concentration)			
Diphacinone	0.9991	1.001	
Chlorophacinone	1.017	0.9995	

Recoveries

Mean recoveries ± standard deviations of chlorophacinone from SRO baits (n = 7 for all validation levels) at the 25 and 150 μg/g levels were 90.7 ± 2.5% and 90.8 ± 2.9%, respectively (Table 2). Mean recoveries of diphacinone ± standard deviations from SRO baits at the 25 and 150 μg/g levels were 93.5 ± 2.9% and 92.3 ± 3.3%, respectively (Table 2). Mean recoveries of chlorophacinone from SRO/wax baits (n = 7 for

Table 2. Fortification of control SRO baits and recoveries

Sample	Concentration of stock solution, μg/mL	Volume, mL	Sample weight, g	Theoretical concentration, μg/g	Observed concentration, μg/g	Receovery, %
			Diphacinone, 150 μg/g			
1	9970	0.0150	1.03	145	129	89.0
2	9970	0.0150	1.02	147	141	95.9
3	9970	0.0150	1.00	150	136	90.7
4	9970	0.0150	1.00	150	144	96.0
5	9970	0.0150	1.01	148	141	95.3
6	9970	0.0150	1.01	148	131	88.5
7	9970	0.0150	1.01	148	134	90.5
					Mean	92.3
					SD	3.3
					CV	3.6%
			Diphacinone, 25 μg/g			
1	997	0.0250	1.04	24.0	22.0	91.7
2	997	0.0250	1.01	24.7	23.3	94.3
3	997	0.0250	1.05	23.7	22.3	94.1
4	997	0.0250	1.01	24.7	24.0	97.2
5	997	0.0250	1.03	24.2	23.3	96.3
6	997	0.0250	1.01	24.7	22.6	91.5
7	997	0.0250	1.01	24.7	22.0	89.1
					Mean	93.5
					SD	2.9
					CV	3.1%
			Chlorophacinone, 150 μg/g			
1	9975	0.0150	1.03	145	130	89.7
2	9975	0.0150	1.02	147	136	92.5
3	9975	0.0150	1.00	150	130	86.7
4	9975	0.0150	1.00	150	143	95.3
5	9975	0.0150	1.01	148	137	92.6
6	9975	0.0150	1.01	148	134	90.5
7	9975	0.0150	1.01	148	131	88.5
					Mean	90.8
					SD	2.9
					CV	3.2%
			Chlorophacinone, 25 μg/g			
1	998	0.0250	1.04	24.0	21.5	89.6
2	998	0.0250	1.01	24.7	22.6	91.5
3	998	0.0250	1.05	23.8	22.0	92.4
4	998	0.0250	1.01	24.7	23.4	94.7
5	998	0.0250	1.03	24.2	21.8	90.1
6	998	0.0250	1.01	24.7	22.1	89.5
7	998	0.0250	1.01	24.7	21.5	87.0
					Mean	90.7
					SD	2.5
					CV	2.8%

Table 3. Fortification of control wax baits and recoveries

Sample	Concentration of stock solution, μg/mL	Volume, mL	Sample weight, g	Theoretical concentration, μg/g	Observed concentration, μg/g	Recovery, %
			Diphacinone, 75 μg/g			
1	1027	0.0760	1.06	73.6	71.1	96.6
2	1027	0.0760	1.06	73.6	72.2	98.1
3	1027	0.0760	1.12	69.7	68.5	98.3
4	1027	0.0760	1.01	77.3	75.2	97.3
5	1027	0.0760	1.01	77.3	75.3	97.4
6	1027	0.0760	1.06	73.6	72.9	99.0
7	1027	0.0760	1.04	75.0	74.6	99.5
					Mean	98.0
					SD	1.0
					CV	1.0%
			Diphacinone, 25 μg/g			
1	1027	0.0250	1.03	24.9	23.1	92.8
2	1027	0.0250	1.05	24.5	22.3	91.0
3	1027	0.0250	1.02	25.2	22.9	90.9
4	1027	0.0250	1.02	25.2	23.6	93.7
5	1027	0.0250	1.01	25.4	23.5	92.5
6	1027	0.0250	1.07	24.0	23.7	98.8
7	1027	0.0250	1.02	25.2	24.1	95.6
					Mean	93.6
					SD	2.8
					CV	3.0%
			Chlorophacinone, 75 μg/g			
1	1024	0.0760	1.07	72.7	73.4	101
2	1024	0.0760	1.03	75.6	76.1	101
3	1024	0.0760	1.09	71.4	73.0	102
4	1024	0.0760	1.24	62.8	61.7	98.2
5	1024	0.0760	1.04	74.8	75.3	101
6	1024	0.0760	1.00	77.8	79.3	102
7	1024	0.0760	1.03	75.6	72.5	95.9
					Mean	100
					SD	2.3
					CV	2.3%
			Chlorophacinone, 25 μg/g			
1	1024	0.0250	1.01	25.3	24.2	95.7
2	997	0.0250	1.02	25.1	23.1	92.0
3	997	0.0250	1.03	24.9	25.5	102
4	997	0.0250	1.05	24.4	24.1	98.8
5	997	0.0250	1.00	25.6	25.9	101
6	997	0.0250	1.09	23.5	23.6	100
7	997	0.0250	1.02	25.1	25.1	100
					Mean	98.5
					SD	3.5
					CV	3.6%

all validation levels) at the 25 and 75 μg/g levels were 98.5 ± 3.5% and 100 ± 2.3%, respectively (Table 3). Mean recoveries of diphacinone from SRO/wax baits at the 25 and 75 μg/g levels were 93.6 ± 2.8% and 98.0 ± 1.0%, respectively (Table 3). Recovery data collected from quality control samples analyzed with actual samples, prepared by various independent contractors, are shown in Table 4. Representative control samples (all components except diphacinone and chlorophacinone) were treated according to the procedures in this method. Recoveries were not significantly different at the levels chosen, which bracket the target concentration. Chromatograms of a commercially prepared chlorophacinone- and diphacinone-fortified SRO and wax bait control samples are shown in Figures 2 and 3 for comparison. As can be seen in Figures 2A and 3A, no chromatographic responses were observed at, or very near, the retention time of chlorophacinone or diphacinone in all control samples. A late-eluting peak was observed that may cause problems in subsequent chromatograms. However this can be avoided by appropriately adjusting run time.

The active ingredient concentration in SRO bait is calculated as follows:

$$\text{Analyte, } \mu\text{g/g} = \frac{A_u}{A_{std}} \times C_{sta} \times \frac{10.00 \text{ mL}}{\text{sample wt (g)}}$$

where A_u = peak area of analyte in sample, A_{std} = peak area of analyte in standard, and C_{std} = concentration of standard (μg/mL).

The active ingredient concentration in wax bait is calculated as follows:

$$\text{Analyte, } \mu\text{g/g} = \frac{A_u}{A_{std}} \times C_{std} \times \frac{25.00 \text{ mL}}{\text{sample wt (g)}}$$

where A_u = peak area of analyte in sample. A_{std} = peak area of analyte in standard, and C_{std} = concentration of standard (μg/mL).

Method Limit of Detection

The method limit of detection (MLOD) was defined as the concentration of chlorophacinone or diphacinone required in the sample to generate a signal equal to 3 times the baseline noise (peak to peak) observed in the chromatogram of the control extract. The MLOD was estimated from the chromatographic response of the analyte in height for extracts of a control bait sample and a control bait sample fortified at 25 μg/g. Under the conditions specified in the method, MLODs for SRO bait were 1.0 μg/g for chlorophacinone and 0.76 μg/g for diphacinone. Under the conditions specified in the method, MLODs for wax bait were 4.2 μg/g for chlorophacinone and 2.8 μg/g for diphacinone.

Conclusions

These methods for analysis of chlorophacinone- or diphacinone-fortified SRO and wax baits are simple, precise, and accurate. They provide high daily sample throughput for both SRO bait (n = 30) and wax bait (n = 20). These methods will be used to support laboratory and field efficacy studies in hopes of registering formulations for rodent control and protection of agriculture and public health.

Acknowledgments

We are grateful to Hacco, Inc., and LiphaTech, Inc., for supplying diphacinone and chlorophacinone and to the California Department of Food and Agriculture for providing funding and control samples.

Table 4. Recoveries of quality control samples of fortified SRO and wax baits determined with actual samples

Analyte	Type of bait	Fortification, μg/g	Number of replicates[a]	Mean recovery, %	Standard deviation	Coefficient of variation, %
Diphacinone	SRO	100	6	94.7	4.9	5.2
Diphacinone	SRO	50	6	95.6	4.5	4.7
Diphacinone	Wax	50	6	101	1.3	1.3
Chlorophacinone	SRO	100	24	90.3	4.8	5.3
Chlorophacinone	SRO	50	12	91.6	7.0	7.6
Chlorophacinone	Wax	50	3	97.5	2.1	2.2

[a] Three quality control samples were assayed for each set of baits analyzed.

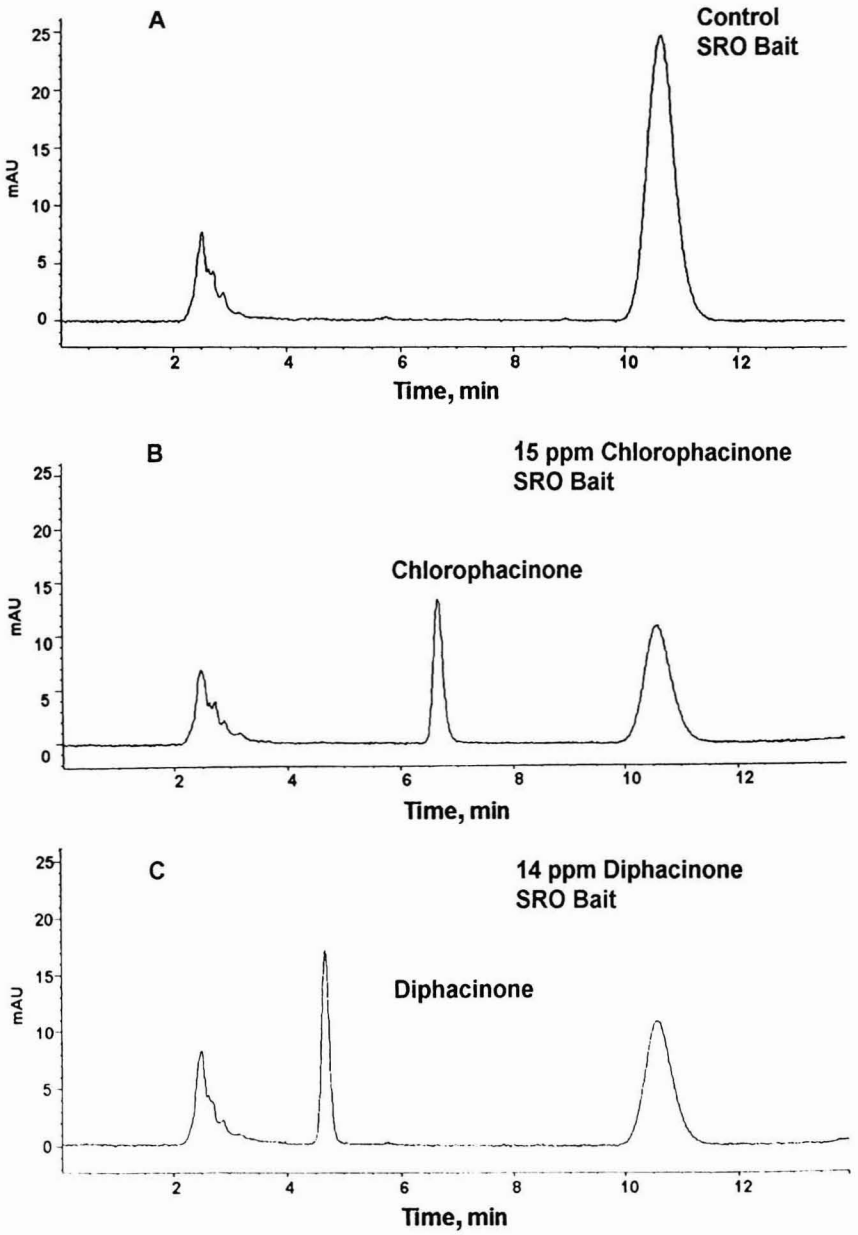

Figure 2. Chromatograms of (A) a blank SRO control sample extract, (B) a 15 μg/g SRO bait chlorophacinone sample extract, and (C) a 14 μg/g SRO bait diphacinone sample extract.

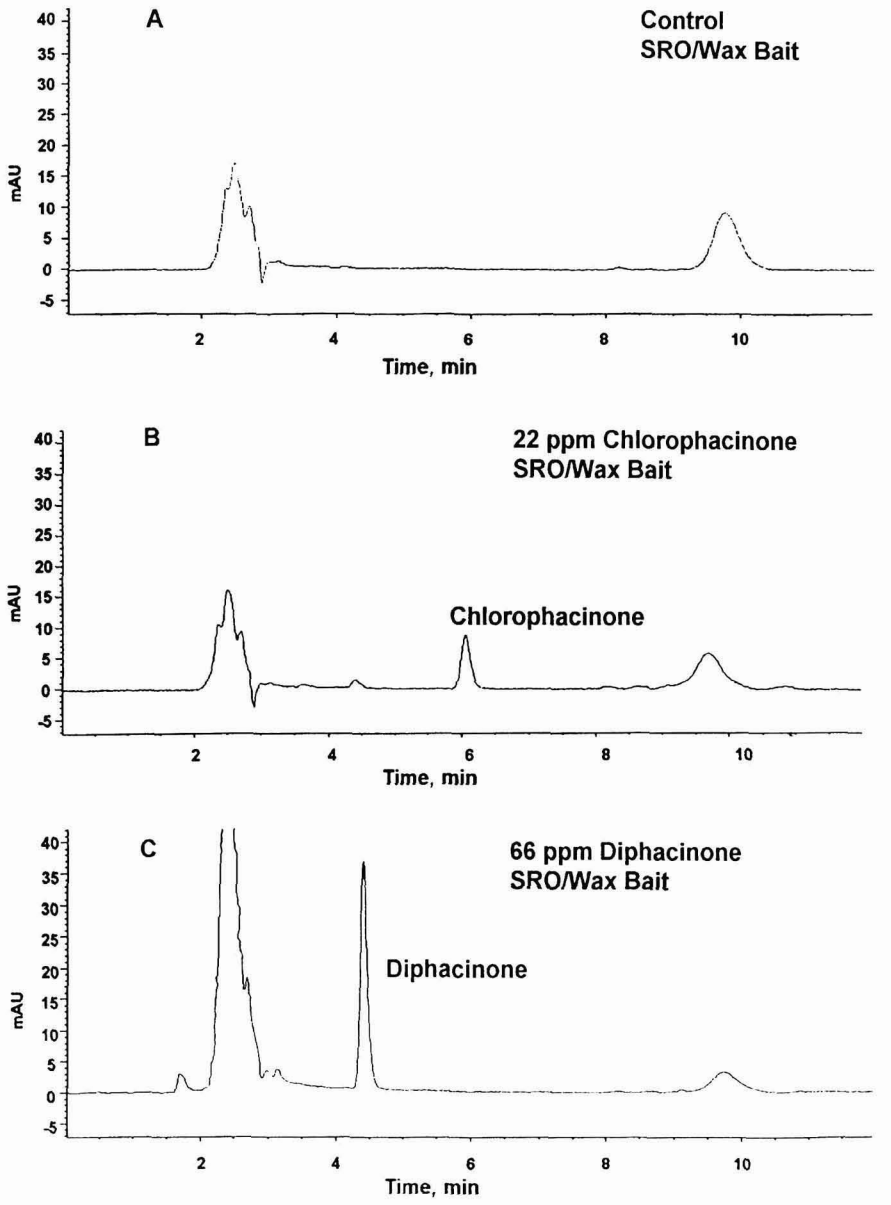

Figure 3. Chromatograms of (A) a blank SRO control sample extract, (B) a 22 μg/g SRO/wax bait chlorophacinone sample extract, and (C) a 66 μg/g SRO/wax bait diphacinone sample extract.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Procedure for Detecting and Confirming Pentobarbital Residues in Dog Food by Gas Chromatography/Mass Spectrometry

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The method described detects and confirms presence of pentobarbital residues in dry, extruded feeds at concentrations of 5–20 ppb. Dried feed is ground to a uniform powder and shaken overnight in methanol. A portion of the methanolic extract is evaporated, and the residue is reconstituted in phosphate-buffered saline. The aqueous extract is cleaned with a solid-phase extraction cartridge designed to extract barbiturate residues from biological matrixes. Dimethyl sulfoxide, tetramethylammonium hydroxide, and iodomethane are added to derivatize pentobarbital. 1,3-Dimethylpentobarbital is then acidified with dilute hydrochloric acid and extracted with isooctane. The organic layer is transferred and evaporated under a stream of nitrogen. The residue is reconstituted in a small volume of ethyl acetate for analysis by gas chromatography/mass spectrometry. The limit of detection is approximately 0.7 ppb. The method was validated with pentobarbital-fortified feed samples containing high concentrations of meat and bone meal.

Pentobarbital is a barbiturate approved by the U.S. Food and Drug Administration for veterinary use as a euthanizing agent (1). Pentobarbital is the preferred euthanizing agent used by animal shelters, racetracks, and veterinarians (2). Disposal of euthanized carcasses may be troublesome and expensive, particularly in metropolitan areas. Rendering these euthanized animal carcasses may be the best disposal option from both a public health and an economic perspective. The carcasses are processed with offal from other sources. The resulting rendered products may contain any contaminants found in the raw materials. Pentobarbital is not degraded by heat rendering and could be present in the final rendered products such as meat and bone meal (MBM) and tallow (3). MBM, tallow, and other rendered products are often used as sources of protein, minerals, and fat by the pet food industry.

This method is designed primarily to analyze pet feeds containing high concentrations of MBM. Not all feed formulations contain MBM as an ingredient. Pet feeds can contain a variety of ingredients from plant and animal sources (4). The method has been tested on samples having label analysis percentages that fall within the following ranges: protein 17–32%; fat 6–20%; fiber 2–9%; moisture, 10–30%.

To determine whether pentobarbital residues are present in dry feed or to gather information on any toxicologic effects, reliable analytical methods are needed (5). The method incorporates solid-phase extraction (SPE) for analysis of barbiturates in biological samples (6, 7). It confirms the presence of pentobarbital residues in dry, extruded animal feeds at concentrations greater than 5 ppb by gas chromatography/mass spectrometry (GC/MS). A full-scan electron ionization mass spectrum of 1,3-dimethylpentobarbital (MW = 254) is shown in Figure 1.

The feed is ground and shaken overnight in methanol. A portion of the extract is evaporated, and the residue is reconstituted in saline. The aqueous extract is cleaned with an SPE cartridge. Iodomethane is added to derivatize pentobarbital. The derivatized sample is acidified and extracted with isooctane. The organic layer is removed and evaporated. The residue is reconstituted in a small volume of ethyl acetate for GC/MS analysis. Six to 9 individual feed samples can be prepared and analyzed during a 24 h period.

The method was validated on commercial feed samples containing high concentrations of MBM. Control feed was fortified with known amounts of pentobarbital. During screening of additional lots of control feed, some lots were found to contain confirmable levels of pentobarbital. Noise levels in the control samples were measured and used to calculate the limit of detection (LOD). An LOD of 0.7 ppb represents a peak response

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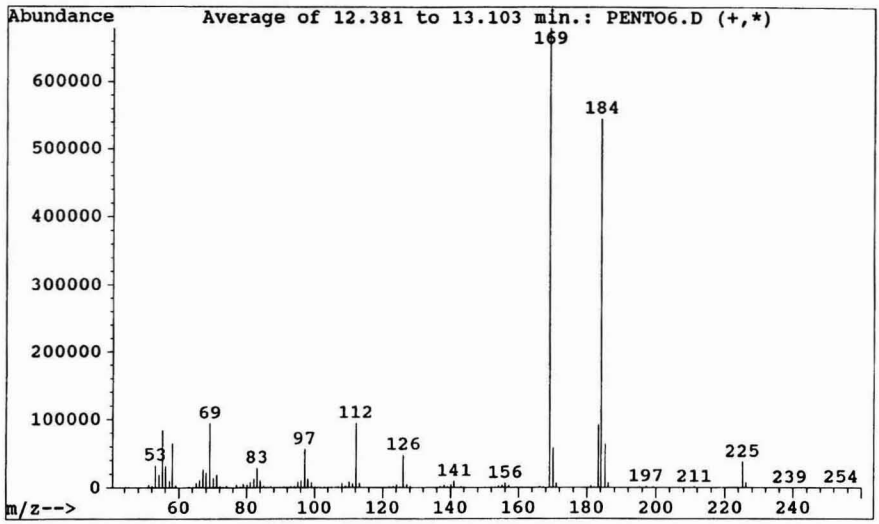


Figure 1. Electron ionization mass spectrum of 1,3-dimethylpentobarbital (MW = 254 amu) showing characteristic fragment ions at *m/z* 169, 184, 185, and 225.

of approximately 3 times the average noise level of a control feed extract.

METHOD

Apparatus

(a) *GC/MS system.*—Hewlett-Packard Series II gas chromatograph equipped with a split/splitless injector; a 2 mm id, 250 μL, quartz-deactivated liner; a Series 5970 mass-selective detector; and a Series 7673A automatic sampler (Hewlett-Packard, Avondale, PA).

(b) *GC column.*—DB-5, 30 m × 0.25 mm id, 0.25 μm film thickness (J & W Scientific, Folsom, CA, or equivalent that meets system suitability requirements). Bake column at 250°C for 8 h before starting each daily analytical run.

(c) *SPE cartridges.*—Bond Elut Certify II, size 10 cc/200 mg, cartridge type LRC (Varian, Harbor City, CA).

(d) *Laboratory blender.*—Waring Model 700B, equipped with borosilicate glass jars, stainless steel cutting blades, and metal screw-on jar lids (Waring Products Corp., Winstead, CT).

(e) *Centrifuge.*—IEC Centra-8R equipped with Model 218 rotor (Damon/IEC Division, Needham, MA).

(f) *Nitrogen evaporator.*—Meyer N-Evap analytical evaporator, Model 111, equipped with Luer adaptors (Organomation Assoc., South Berlin, MA).

(g) *Rotary evaporator.*—Buchi Model EL-131 equipped with Model 461 water bath (Buchi Laboratoriums Technik AG, Flawil, Switzerland).

(h) *Digital platform shaker.*—Innova 2100 (New Brunswick Scientific, Edison, NJ).

(i) *Syringes.*—Use a dedicated syringe for each derivatization reagent; Hamilton series 700 standard microliter syringes with fixed point No. 2 needles, size/model No./catalogue No.: 100 μL/710/80630, 25 μL/702/80400, and 50 μL/705/80500 (Hamilton, Reno, NV). Also B-D Multifit 2 cc glass syringe with Luer-Lok tip; syringe, catalogue No. 2440; needle, catalogue No. 1047 (Becton-Dickinson and Co., Rutherford, NJ).

(j) *Vacuum manifold.*—Visiprep 12-port SPE vacuum manifold, catalogue No. 5-7030 (Supelco, Bellefonte, PA).

Reagents

(a) *Solvents.*—UV spectrophotometric grade ethyl acetate, hexane, isooctane, methanol.

(b) *Water.*—Distilled, deionized.

(c) *Dimethyl sulfoxide (DMSO).*—Silylation grade, catalogue No. 20684 (Pierce Chemical Co., Rockford, IL), or equivalent.

(d) *Hydrochloric acid (HCl).*—Concentrated.

(e) *Iodomethane.*—Contains 0.3% metallic copper as a preservative, catalogue No. I-8504 (Sigma Chemical Co., St. Louis, MO), or equivalent.

(f) *Stearic acid methyl ester (methyl stearate).*—Catalogue No. M-5376 (Sigma), or equivalent.

(g) *Pentobarbital standard solution, 98% pure.*—Prepared (w/v) in methanol at 1.0 mg/mL. Catalogue No. P-3393 (Sigma), or equivalent.

(h) *Phosphate buffered saline (PBS), pH 7.4.*—Catalogue No. 16-006Y (BioWhittaker, Walkersville, MD), or equivalent.

(i) *Sodium sulfate.*—Anhydrous powder.

(j) *Tetramethylammonium hydroxide (TMAH).*—25% in methanol, catalogue No. T-0280 (Sigma), or equivalent.

Solutions

(a) *Alkylation reagent.*—TMAH–DMSO, 1 + 20, prepare daily.

(b) *Hexane–ethyl acetate.*—95 + 5, prepare daily.

(c) *Hexane–ethyl acetate.*—75 + 25, prepare daily.

(d) *Hydrochloric acid, 0.1N.*—Dilute 1N HCl, 1 + 10 (stable for 1 year).

(e) *Sodium acetate buffer.*—100 mM, pH 7. Add 13.6 g sodium acetate to 1 L glass-stoppered, graduated flask. Add deionized water to a level slightly below 1 L. Shake well to dissolve crystals. Adjust the pH to 7.0 (±0.1) with 1N NaOH or 1N HCl. Add deionized water to 1 L mark. Store at 4°C (stable for 3 months).

Standard Solutions

(a) *Pentobarbital stock standard (SS-10, 10 μg/mL).*—Transfer 1.0 mL stock into 100 mL volumetric flask. Dilute to volume with methanol. Store at ≤0°C (stable for 12 months).

(b) *Pentobarbital working standard (WS-0.1, 0.1 μg/mL).*—Take 1.0 mL SS-10 into 100 mL volumetric flask. Dilute to volume with methanol. Store at ≤0°C (stable for 6 months).

(c) *Unextracted standard (UES, equivalent to 10 ppb in feed).*—Add 500 μL WS-0.1 to a disposable screw-cap glass centrifuge tube. Evaporate solution to dryness. Derivatize and analyze along with the extracted sample.

Sample Treatment

(a) *Sampling.*—Start with a 5 lb (or larger) bag of feed, sample from at least 2 places in the bag, and mix the 2 samples before filling blender jar.

(b) *Feed preparation.*—Grind dry extruded pet feed sample to uniform powder in laboratory blender. A specific sieve size is not required; however, resulting uniform powder should be easily weighed, transferred, and solvated in methanol.

(c) *Feed extraction.*—Weigh 10 g uniformly ground powder into a bottle with a Teflon-lined cap. To prepare fortified samples, add appropriate amounts (2.0, 1.0, or 0.5 mL) of WS-0.1 to weighed sample. These volumes are equivalent to 20, 10, and 5 ppb in feed. Allow fortification solvent to evaporate completely in hood. Add 100 mL methanol to each sample. Cap bottle tightly and place on orbital shaker for overnight mechanical extraction. Allow feed to settle and pour upper layer into a polypropylene centrifuge tube. Centrifuge sample at room temperature (1340 × *g*, 10 min) or until methanol appears clear and a small pellet forms at the bottom.

Sample Cleanup

(a) *Preparation of PBS extract.*—Remove 50 mL clear methanol extract without disturbing pellet and transfer to a 200 mL pear-shaped flask with a ground-glass stopper. Evaporate extract in a rotary evaporator (100 rpm, 20° ± 5°C) until an oily dark residue remains. Dissolve residue in 5 mL PBS and 2 mL sodium acetate buffer. A yellow, waxy film will remain on the interior of the pear-shaped flask. Using a pipet, transfer all aqueous extract to a 10 mL glass centrifuge tube. Centrifuge at room temperature (1930 × *g*, 10 min). Three layers will form: a soft yellow fat layer, a clear methanol layer, and a solid pellet. Set glass tubes aside.

(b) *SPE.*—Condition cartridges with 2 mL methanol followed by 2 mL 0.1M sodium acetate buffer, pH 7.0. A flow rate of 1–2 drops/s should be maintained. Do not allow SPE to dry. Return to aqueous extracts in glass tubes. Remove all traces of upper yellow fat layer with a cotton-tipped swab. Then, without disturbing solid pellet at the bottom, use Pasteur pipet to remove all the clear liquid. Transfer this liquid directly to prepared SPE cartridge. Reduce vacuum to ca 2–3 in. Hg and slowly draw extract through SPE until all extract has been loaded at a flow of ca 1 drop/s. Wash charged SPE cartridge with 1 mL 0.1M sodium acetate buffer, pH 7.0. Dry cartridge under full vacuum for 5 min. Wash cartridge again with 2.0 mL hexane–ethyl acetate (95 + 5). Open SPE manifold and wipe excess liquid from tips of guide needles. Elute pentobarbital with 2.0 mL hexane–ethyl acetate (75 + 25) into labeled 15 mL screw-cap glass centrifuge tubes. Dry cartridges under full vacuum for 1 min. Place sample tubes and previously prepared UES in nitrogen evaporator and evaporate under a stream of nitrogen at room temperature.

Sample Derivatization

(a) *Alkylation.*—Prepare sufficient alkylation reagent for all specimens in batch. Recommended amount for a batch of 12 tubes is prepared by adding 0.1 mL (100 μL) TMAH (25% in MeOH) to 2.0 mL DMSO. Cap vial and shake alkylation reagent briefly on a Vortex mixer. To each tube containing dried residue, add 100 μL alkylation reagent, TMAH–DMSO (1 + 20), and shake on Vortex mixer for a minimum of 30 s. Let sit for 2 min.

(b) *Methylation.*—Add 25 μL iodomethane to each tube and shake briefly on Vortex mixer as iodomethane is added to the bottom of each tube. Cap tubes and let sit 5 min before proceeding.

(c) *Extraction.*—Add 0.4 mL 0.1N HCl to each tube. Add 2 mL isooctane to each tube. Cap tubes and shake on Vortex mixer for ca 2 min with frequent stopping and starting of Vortex mixer. Centrifuge tubes at 860 $\times g$ for 5 min. Prepare a dry ice–isopropyl alcohol bath. Place tubes in bath to freeze lower aqueous layer. Decant upper layer to a 5 mL clean, disposable, conical glass tube. Evaporate organic solvent to dryness at room temperature under a stream of nitrogen. Remove tubes as soon as they are dry. Reconstitute derivatized extracts in 100 μL ethyl acetate, shake on Vortex mixer briefly, and transfer to GC vials containing glass inserts to accommodate the small volume. Analyze samples within 24 h.

Chromatographic Conditions

(a) *GC conditions.*—The carrier gas is helium, linear velocity is set at 29 cm/s at 40°C, the injector temperature is 270°C, and a splitless 1 μL injection is used. The temperature program is 40°C for 1 min, 30°C/min to 140°C, 6°C/min to 190°C, hold for 3 min, and 30°C/min to 250°C, hold for 12 min.

(b) *MS conditions.*—Interface temperature is 280°C. Selected ion monitoring (SIM) mode is set for 4 ions diagnostic of 1,3-dimethylpentobarbital, *m/z* 225, 185, 184, and 169. Structures and proposed fragmentations of these ions are shown in Figures 2 and 3. Adjust dwell time to get at least 2.0 cycles/s.

System Suitability

(a) *Instrument check.*—Methyl stearate test mix is prepared at a concentration of 1 ng/μL in methanol and injected directly onto GC/MS system. Ions *m/z* 298 and 299 are monitored. Peak-to-peak signal-to-noise (S/N) ratio must be greater than 75. The temperature program is 40°C for 1 min, 30°C/min to 100°C, then 30°C/min to 250°C, hold for 10 min. GC settings are as follows: injector temperature, 195°C; MS interface, 200°C.

(b) *Method check.*—Establish that derivatization reagents and other aspects of laboratory procedure are performing within acceptable limits. Calculate S/N ratio using UES. Minimum S/N ratio should be ≥ 150.

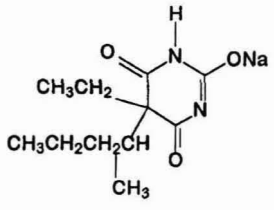


Figure 2. Structure of pentobarbital sodium salt (MW = 248 amu).

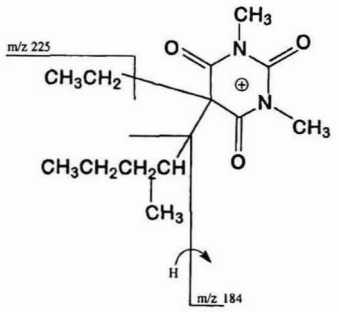


Figure 3. Structure of 1,3-dimethylpentobarbital (MW = 254 amu) in electron ionization showing proposed fragmentation of characteristic fragment ions *m/z* 225 and *m/z* 184. Ion *m/z* 185 is thought to be an isotope cluster. The exact structure cannot be accounted for, however, because cleavage and rearrangement are involved.

(c) *Baseline check.*—Use an injection of ethyl acetate to verify baseline stability at the start of each day.

(d) *Tailing factor.*—Using UES injection, calculate tailing factor as described in the chromatography section of the current U.S. Pharmacopeia (8). It should be ≤ 1.2.

Confirmation Criteria

If a test sample passes all criteria it is confirmed. If the test sample does not pass the criterion, it is designated as "fails to confirm."

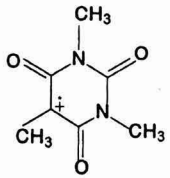
(a) *Ion retention times.*—These should fall within 3 scans of the retention times of the pentobarbital UES included with each batch. To determine this limit, multiply the number of ions being monitored by the dwell time per ion times 3, or the number of scans allowed.

(b) *Relative abundances of monitored ions.*—Calculate relative abundances of *m/z* 184, 185, and 225 ions with respect to abundance of *m/z* 169 ion for each unfortified control, fortified control, and suspect feed sample run. For a given sample to be within acceptable limits of the criterion, the relative abundances of the sample *m/z* 184, 185, and 225 ions must be within ±10% of the average relative abundances of the same ions measured for derivatized UES.

(c) *Validation.*—For a day's analysis of samples to be considered valid, control extract must fail to confirm, and sample fortified with known amounts of pentobarbital must be confirmed.

Results and Discussion

This method was validated by analysis of a minimum of 10 unfortified control feed samples and sets of



m/z = 169

Figure 4. Proposed structure of the base peak ion *m/z* 169.

10 fortified control feed samples containing pentobarbital at 5, 10, and 20 ppb. Figure 4 shows selected ion traces for a 1,3-dimethylpentobarbital standard. Figure 5 shows a fortified control feed sample, and Figure 6 shows an unfortified control feed sample extract. All unfortified control feed samples were negative; that is, they did not confirm. All fortified control feed samples were confirmed. Table 1 lists ion abundances for SIM analysis of standards, unfortified controls, and control feed samples fortified at 10 ppb. Integration was forced for unfortified control feed samples at expected retention time of pentobarbital. Unfortified control feed samples had a small *m/z* 169 peak

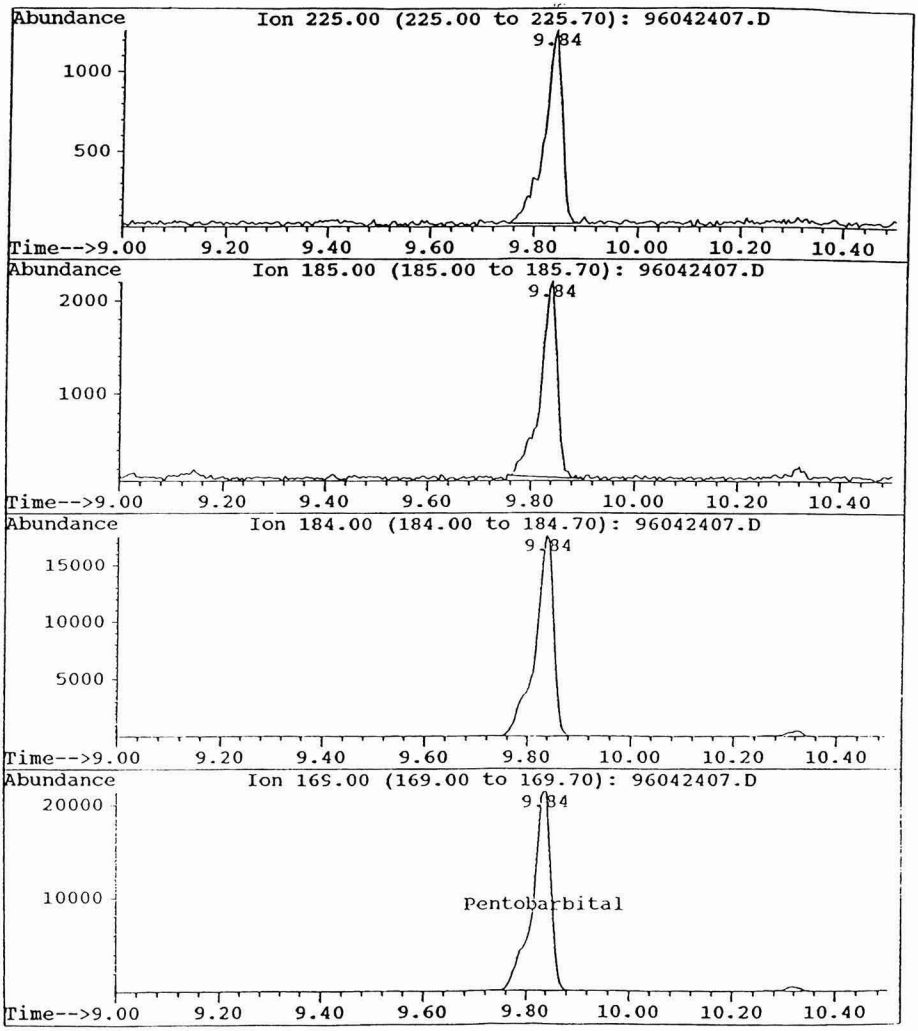


Figure 5. Selected ion chromatograms for *m/z* 225, 185, 184, and 169 ions arising from derivatized pentobarbital standard equivalent to 10 ppb in feed.

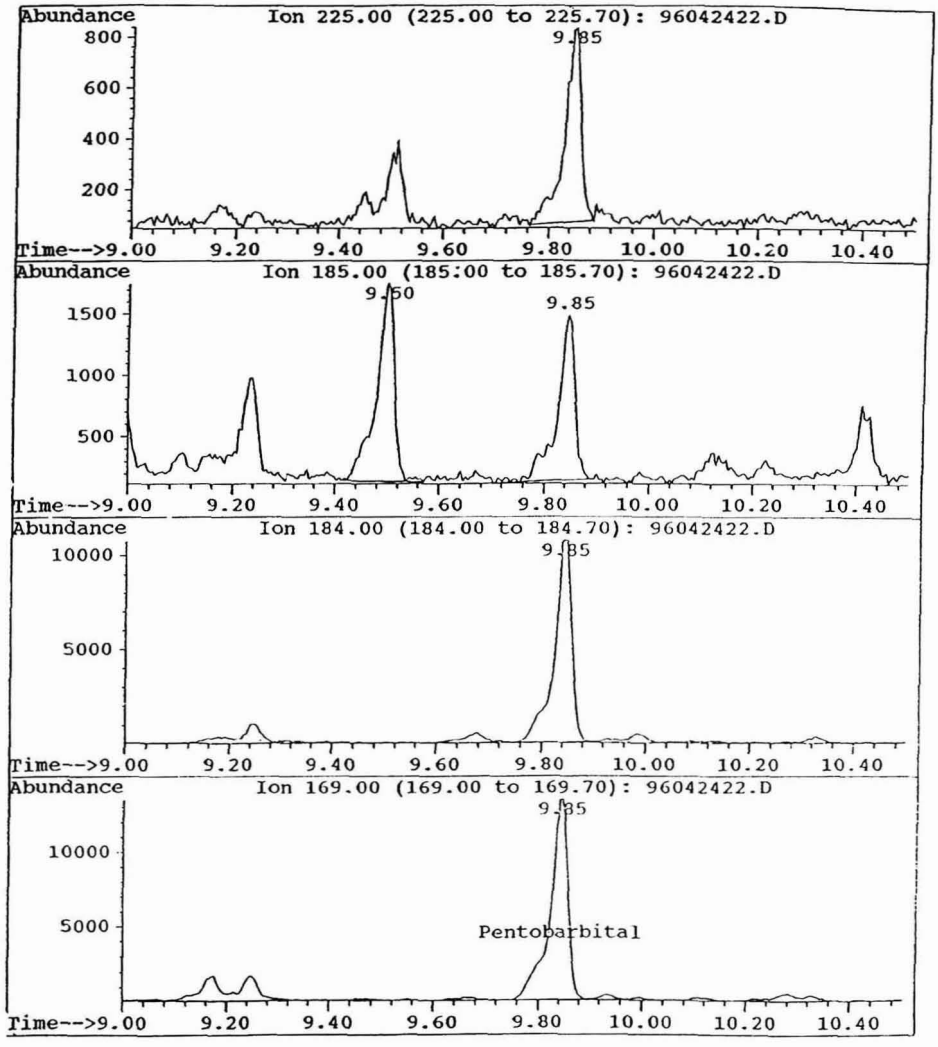


Figure 6. Selected ion chromatograms for *m/z* 225, 185, 184, and 169 ions arising from derivatized pentobarbital extracted from a 10 ppb fortified control feed sample.

Table 1. *Average relative abundances of fragments from* 1,3-dimethylpentobarbital (mean ± standard error)

Sample	*n*	*m/z* 169/169	*m/z* 225/169	*m/z* 185/169	*m/z* 184/169
Standard (10 ppb)	5	100	4.4 ± 0.24	8.7 ± 0.13	76.0 ± 1.13
Acceptance limits		Base peak	0 < X < 14.8	0 < X < 18.7	66–86
Control feed	10	100	1.68 ± 0.61	12.0 ± 2.69	14.1 ± 2.62
Fortified (10 ppb)	10	100	4.5 ± 0.20	9.2 ± 0.25	76.0 ± 0.89
Commercial feed A	3	100	5.0 ± 0.29	9.9 ± 0.47	7.85 ± 4.5
Commercial feed B	3	100	5.1 ± 0.27	9.5 ± 0.45	78.1 ± 2.67

(<0.5% of standard) at the retention time of pentobarbital, which may be due to carryover. These unfortified feed samples did not meet the ion-abundance-matching criteria and were not confirmed. All fortified control feed samples met both retention time and ion-abundance-matching criteria and were confirmed. Table 1 also lists confirmation results for 2 commercially available dog foods. Both formulations are labeled as containing MBM as one of the top 5 ingredients. These feeds were analyzed on 3 different days to verify presence of pentobarbital. Figure 7 is a solvent blank showing a small interference peak at the expected retention time of pentobarbital, indicating a little carryover. Figure 8 shows selected ion traces for a suspect feed sample that was confirmed by this method.

Conclusions

The method reliably confirmed the presence of pentobarbital in dry extruded pet feeds containing significant concentrations of MBM. Using a control feed that previously had been screened and known to be free from pentobarbital residues, samples were fortified at 5, 10, and 20 ppb, and all fortified samples were confirmed. All companion control samples failed to confirm. Method performance was validated by analyzing several lots of commercial feed samples suspected of containing pentobarbital residues. In all suspect cases, pentobarbital residues were confirmed. This method is well suited for screening or surveying large numbers of samples. Sample results easily can be screened, and positive results immediately can be confirmed.

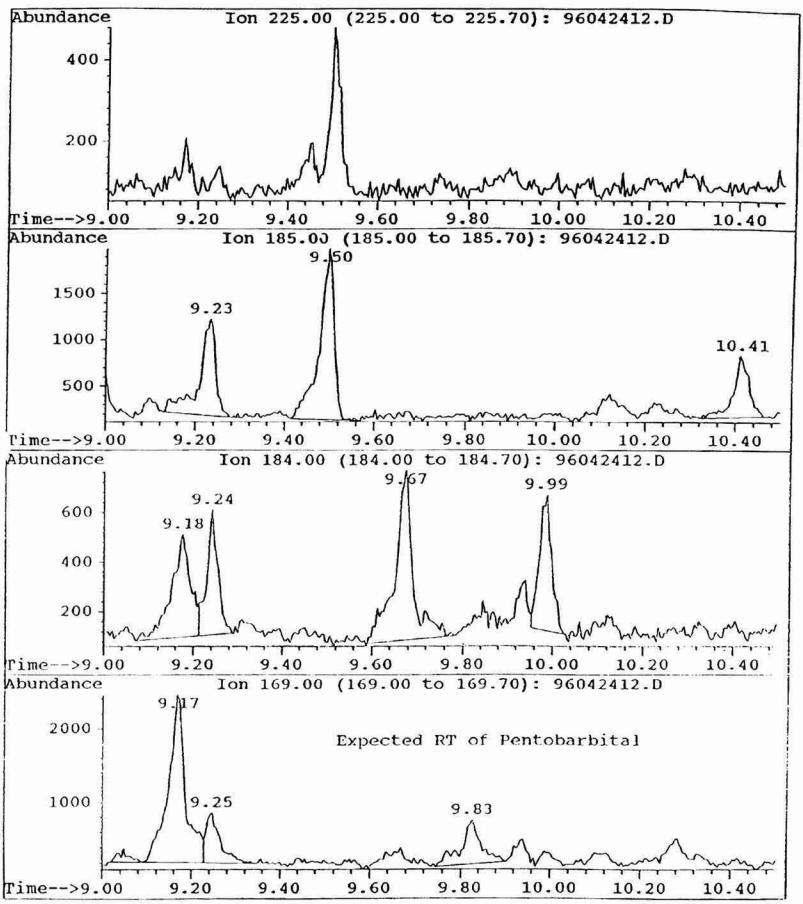


Figure 7. Selected ion chromatograms for *m/z* 225, 185, 184, and 169 ions arising from unfortified control feed extract.

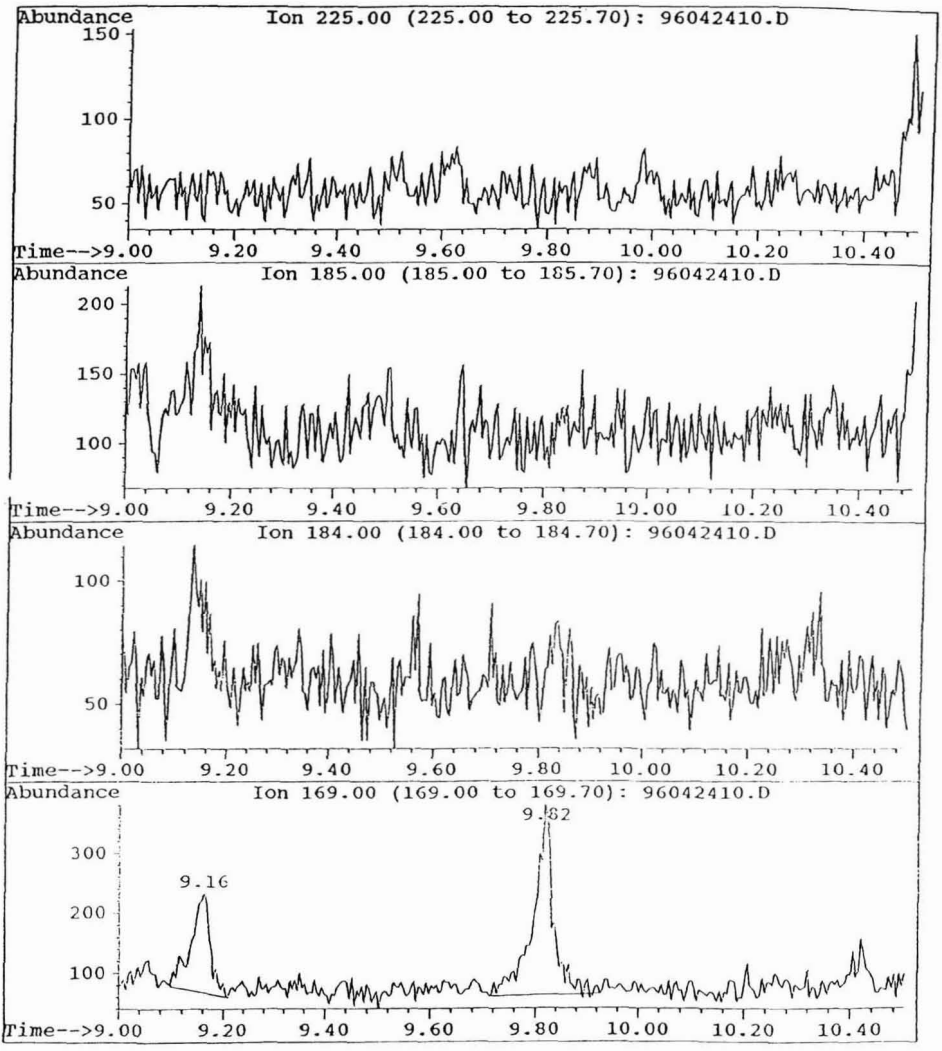


Figure 8. Selected ion chromatograms for *m/z* 225, 185, 184 and 169 ions arising from a processed solvent blank, showing a small interference peak at *m/z* 169 close to the expected retention time of pentobarbital.

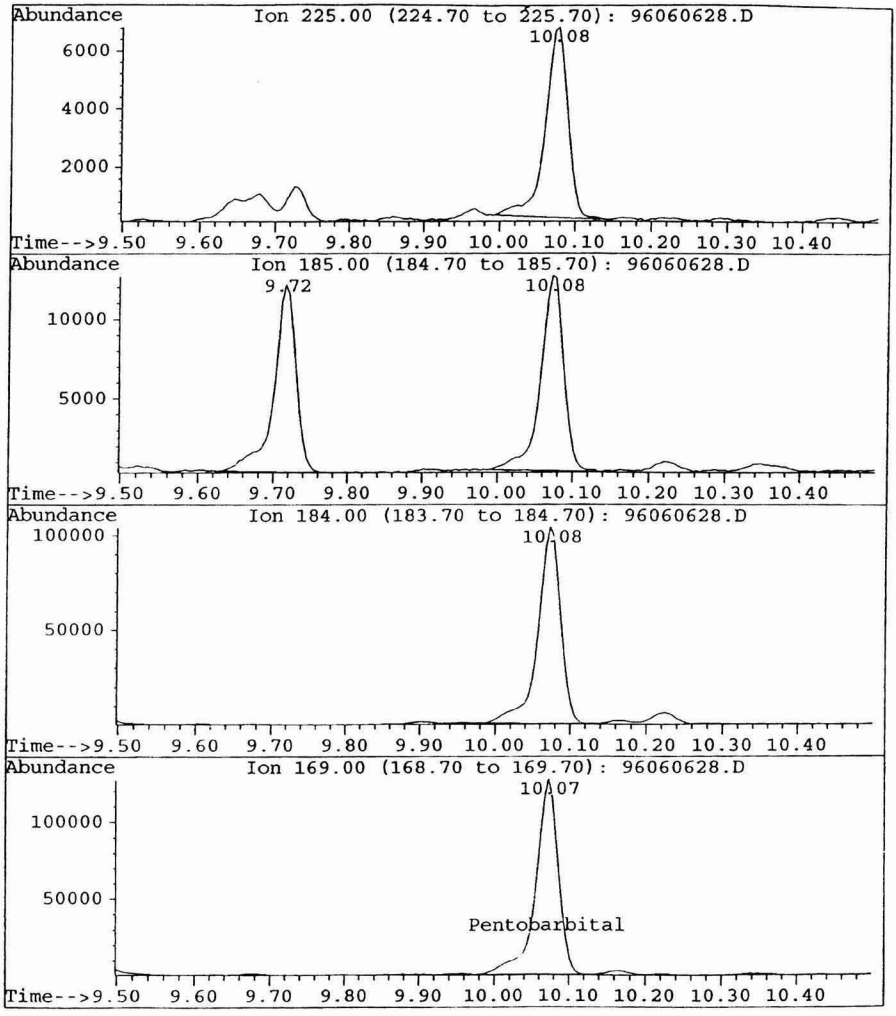


Figure 9. Selected ion chromatograms for *m/z* 225, 185, 184, and 169 ions arising from derivatized pentobarbital in an extract of a suspect feed sample confirming presence of pentobarbital.

Acknowledgments

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A Rapid and Selective Method for Determining Potential Nitrosating Agents in Cosmetic Products by Chemiluminescence Detection of Nitric Oxide

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A method was developed for rapid and selective determination of potential nitrosating agents at the part-per-billion level in cosmetic products. These compounds are chemically reduced to nitric oxide, which is determined by its chemiluminescent reaction with ozone. Suspended materials and colors in cosmetic products do not interfere. Hence their removal before analysis is not required. A detection limit of 33 ppb, calculated as nitrite, was obtained. No false-positive interferences were observed from antifoaming agents, several *N*-nitroso compounds, and nitrate up to 20 ppm. Among cosmetic products surveyed, potential nitrosating agents were found at levels ranging from 113 to 5021 ppb. No consistent relationship was found between levels of potential nitrosating agents and *N*-nitrosamines in the same products. However, the highest levels of nitrosating agents were most often associated with the highest levels of *N*-nitrosamines known to be present in the products.

N-Nitrosamines are known to be carcinogenic in a variety of animal species (1). They are formed by the reaction of amines and amine derivatives with a nitrosating agent, usually derived from nitrite.

Cosmetic products are formulated with various amines and their derivatives. Several *N*-nitrosamines have been identified in commercial products (2). *N*-Nitrosamines are either present in raw materials or are formed in the products by the reaction of amines with nitrosating agents when precursor-containing raw materials are formulated. The primary source of *N*-nitrosamine contamination is formation in the cosmetic products themselves from precursors (3). Nitrosating agents such as nitrite and nitrite derivatives are not added intentionally to cosmetic products. However, nitrite and nitrate are ubiquitous in the environment and may be present as impurities in cosmetic ingredients such as inorganic raw materials and pigments (4). Cosmetic raw materials stored in metal drums treated with anticorrosive agents such as nitrite may also become contaminated.

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It would be advantageous to have a simple, rapid, and selective method to detect nitrosating agents such as nitrite at part-per-billion levels in cosmetic products to assess the potential for *N*-nitrosamine formation. Such a method could be used to screen a large number of products, and those products with the highest levels of potential nitrosating agents could be analyzed further for *N*-nitrosamines.

Methods for determining nitrite in foods and biological systems (5) include a colorimetric method based on the Griess reaction (6), a polarographic method (7), a gas chromatographic method after derivatization (8), liquid chromatography (LC) using different detectors (9), and chemiluminescence detection after reduction of nitrite to nitric oxide by suitable reductants (10, 11). The procedure of Walters et al. (12) for determining nitrite in food involved acidifying samples with acetic acid and measuring the nitric oxide generated with a chemiluminescence detector. The method was potentially at least one order of magnitude more sensitive than colorimetric methods, because chemiluminescence detection of nitric oxide is specific and more sensitive than detection methods for colorimetric methods. Water, however, decreased the method's response, thereby limiting applicability.

Only colorimetric methods have been reported for determining nitrite in cosmetic raw materials. The method of Rao Gadde and Patel (13) involved the reaction of nitrite with sulfanilamide in acidic solution to form a diazonium salt. When the salt was coupled with an aromatic amine such as *N*-1-naphthylenediamine, a highly colored azo compound was produced that could be measured by its absorbance at 543 nm. The method had a limit of determination in the part-per-million range.

The method of Rosenberg et al. (14) involved diazotization of sulfanilic acid by nitrite followed by coupling with either *m*-aminophenol or *m*-dimethylaminophenol to form a colored azo dye, which was measured spectrophotometrically. The method had a detection limit

of 10 ppb but was affected by various interferences from cosmetic raw materials such as pigments, gelling agents such as methylcellulose, and polyethylene glycol derivatives. Thus the method required elaborate cleanup of cosmetic matrixes.

The use of chemiluminescence for determining nitrite was extended to aqueous systems by Cox (15). In the method of Cox, nitrite was selectively reduced under mild conditions with sodium iodide as a reductant in the weakly acidic medium of aqueous acetic acid. The nitric oxide produced was removed and transferred to a stream of helium into a chemiluminescence analyzer for measurement. The method provided greater sensitivity, accuracy, and precision than colorimetric methods. It did not require removal of suspended material or color, which interfered with colorimetric methods.

In this study, the method of Cox was evaluated to assess the effectiveness of a chemiluminescence method for determining nitrite and other potential nitrosating agents in cosmetic products. Results of a survey of cosmetic products for potential nitrosating agents are presented.

METHOD

N-Nitrosamines are suspected carcinogens. Extreme care should be exercised when handling them.

Apparatus

(a) *Nitric oxide analyzer.*—Thermal energy analyzer (TEA) Model 502 (Thermedics Detection, Inc., Chelmsford, MA) operated under the following conditions: pyrolyzer temperature, 550°C; total TEA reaction chamber pressure, 0.5 torr; carrier gas, argon; cold traps, 2 glass cold traps in dry ice/acetone bath (−70°C).

(b) *Degassing apparatus.*—Similar to that used by Drescher and Frank (16); coarse fritted glass dispersion tube; 24/40 male fitting connected to a 25 mm × 17 cm test tube.

(c) *Adsorption trap.*—10 cm × 6 mm id stainless steel tubing packed with anhydrous sodium carbonate and fitted with glass wool plugs in each end. Unit is fitted with Swagelok connectors. Adsorption trap is heated at 150°C overnight before use.

(d) *Electronic integrator.*—Model 3390A (Hewlett-Packard, Palo Alto, CA).

(e) *Gas stream filter.*—Thermedics Detection, Inc.

Reagents

(a) *Sodium iodide, silver nitrate, and pyridine.*—ACS grade (Fisher Scientific Co., Fair Lawn, NJ).

(b) *Glacial acetic acid.*—ACS grade (J.T. Baker, Inc., Phillipsburg, NJ).

(c) *Sodium nitrite, n-decyl alcohol, and n-butyl nitrite.*—ACS grade (Aldrich Chemical Co., Milwaukee, WI).

(d) *Deionized water.*—Water purified through a Milli-Q water system (Millipore Co., Bedford, MA).

(e) *Sodium chloride.*—ACS Grade (EM Science, Gibbstown, NJ).

(f) *Antifoam A.*—Concentrate (Sigma Chemical Co., St. Louis, MO).

(g) *N-Nitrosodimethylamine, N-nitrosomorpholine, N-nitrosodiethanolamine, and N-nitrosodiisopropanolamine.*—Thermedics Detection, Inc.

(h) *2-Ethylhexyl 4-(N-methyl-N-nitrosamino) benzoate.*—Synthesized in this laboratory (17).

(i) *Sodium iodide solution.*—Prepare 0.1M solution by dissolving 0.75 g sodium iodide in 50 mL deionized water. Prepare daily and store at 4°C.

(j) *$NaNO_2$ stock solution.*—10 μg/mL. Accurately weigh ca 10 mg $NaNO_2$ into 1 L volumetric flask, dilute to volume with deionized water, and mix.

(k) *$NaNO_2$ standard solution.*—10 ng/mL. Pipet 1 mL $NaNO_2$ stock solution into 1 L volumetric flask, dilute to volume with deionized water, and mix.

(l) *n-Decyl nitrite.*—Prepare by reaction of *n*-decyl alcohol with nitrosyl chloride according to the procedure of Bouveault and Wahl (18).

Analysis

(a) *Preparation of test solution.*—Disperse ca 1 g cosmetic product in 20 mL deionized water by heating on a steam bath until mixture is uniform.

(b) *Determination of potential nitrosating agents.*—Connect, in series, carrier gas line, degassing apparatus, and adsorption trap to TEA inlet with 1/4 in. od Teflon tubing. (A diagram of this arrangement is shown in reference 16). Operate TEA with conditions given for nitric oxide analyzer, *Apparatus* (a). Turn stopcocks to bypass position; remove test tube; and add 5 mL sodium nitrite standard solution, 15 mL deionized water, and 1 mL 0.1M sodium iodide solution; and mix thoroughly. Add 3 mL glacial acetic acid to mixture and immediately place test tube on degassing apparatus. Turn stopcocks to allow carrier gas to pass through reaction mixture. Continue purge until no further evolution of nitric oxide is observed on integrator. Return stopcocks to bypass position. In a clean test tube, analyze 5 mL dilute cosmetic suspension containing 200 μL *n*-decyl alcohol to control foaming by the same procedure. Calculate amount of potential nitrosating agents present by using peak area from test portion and average areas of 2 $NaNO_2$ standard analyses as follows:

Nitrosating agents,

$$\text{ppb} = \frac{A_{\text{sam}} \times C_{\text{std}} \times \text{Mw NO}_2}{A_{\text{sam}} \times W_s \times \text{Mw NaNO}_2}$$

where A_{sam} = peak area of analyte, C_{std} = concentration of sodium nitrite standard solution

(ng/mL), Mw NO_2^- = molecular weight of nitrite, A_{std} = average peak area for 2 $NaNO_2$ standard solution determinations (sodium nitrite peak areas should differ by <10%), W_s = weight of test portion (g), and Mw $NaNO_2$ = molecular weight of sodium nitrite.

Results and Discussion

To identify cosmetic products with high potential for *N*-nitrosamine contamination, a rapid and selective method was developed to determine the concentration of potential nitrosating agents. If levels of nitrosating agents correlate with levels of *N*-nitrosamines in the products, then the method could be used to rapidly identify products most likely to contain *N*-nitrosamines.

Although a number of reactants can be used to liberate nitric oxide from nitrite, iodide ion in weakly acidic medium was selected. The concentration of sodium iodide/acetic acid solution was optimized to maximize rapid yield of nitric oxide. Glacial acetic acid by itself did not generate sufficient nitric oxide from sodium nitrite to give a chemiluminescent response. When cosmetic products were analyzed for nitrosating agents, some produced a broad peak, which was likely due to matrix interaction effects; therefore peak area was chosen for quantitation. The chemiluminescent response for nitric oxide released from a standard sodium nitrite solution and a cosmetic product is shown in Figure 1.

The method was evaluated for selectivity, sensitivity, and reproducibility. Four determinations of a 50 ppb sodium nitrite standard were made, and a relative standard deviation of 10% was obtained. The detection limit of the method was 33 ppb, calculated as nitrite.

Because the method is based on detection of nitric oxide, any compound generating nitric oxide under the experimental conditions used will give a response. Therefore, the method was evaluated for selectivity by analyzing several compounds that may also give a positive response. At a 10 ppm excess of nitrate (as the silver salt), no chemiluminescent response was observed. Determination of *N*-nitroso compounds by chemiluminescent determination of nitric oxide has been reported previously (17). To determine if *N*-nitrosamines found in cosmetic products would give a response by this method, *N*-nitrosodiethanolamine (NDELA), *N*-nitrosodiisopropanolamine, *N*-nitrosodimethylamine, *N*-nitrosomorpholine, and 2-ethylhexyl 4-(*N*-methyl-*N*-nitrosamino) benzoate (NMPABAO) were analyzed at levels up to 20 ppm; no responses were observed.

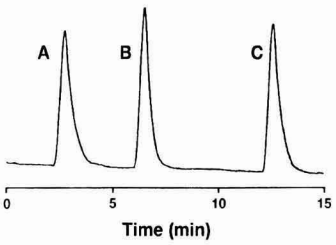

Figure 1. Chemiluminescence (TEA) response of 70 ng sodium nitrite (A and C) and cosmetic test solution (B).

Alkyl nitrites may be present in cosmetic products as a result of the reaction between alcohols and nitrite or other nitrosating agent (20). Alkyl nitrites, in turn, also can act as nitrosating agents (21). Water-soluble alkyl nitrites such as butyl nitrite give a positive response to a chemiluminescence detection system (11). Butyl nitrite added to a cosmetic matrix also gave a positive chemiluminescence response when analyzed by the method described in this paper. Because cosmetic products may contain long-chain fatty alcohols, nitrite esters may also be present if nitrosating agents are present. To determine if a chemiluminescence response observed from a cosmetic product analyzed by the method described in this paper would also include the response to any long-chain alkyl nitrites present, *n*-decyl nitrite was synthesized and added to a cosmetic emulsion. No chemiluminescent response was observed for the long-chain alkyl nitrite, most likely because of the insolubility of *n*-decyl nitrite in the cleavage reagent.

Most cosmetic products contain emulsifiers or surfactants that during analysis generate foams that can interfere with determination of nitrosating agents. Antifoaming agents such as sodium chloride, *n*-decyl alcohol, and Antifoam A were tested to ensure they did not produce false-positive chemiluminescent responses under the experimental conditions. No false-positive responses were observed.

The cosmetic preservative 2-bromo-2-nitro-1,3-propanediol (BNPD) decomposes and releases nitrite and formaldehyde under alkaline pH and is an effective indirect nitrosating agent (22). NDELA is readily formed by the reaction of diethanolamine with nitrite formed by the decomposition of BNPD (23). The highest levels of NDELA in cosmetic products usually are associated with products also containing the preservative BNPD (3). Because of the potential for nitrite release from BNPD, BNPD was analyzed by the method described to determine if it would give a chemiluminescent response. As expected, no response was observed, because BNPD is stable at acid pH (22).

The method was validated through recovery studies on 2 cosmetic product types: a lotion and a cream. Products were fortified with sodium nitrite at 47, 70, and 93 ppb. Results are shown in Table 1. Recoveries, calculated as nitrite, from the cream averaged 82% (standard deviation [SD], 2.9). Recoveries from the lotion averaged 87% (SD, 2.9).

Sixteen cosmetic products previously analyzed and found to contain NDELA and/or NMPABAO were analyzed for total potential nitrosating agents to deter-

Table 1. Recovery of NO_2^- from cream and lotion

Sample	NO_2^- Added, ppb	NO_2^- Found, ppb	NO_2^- Recovery, %
Cream	47	37, 37, 37	79, 79, 79
	70	57, 59, 58	81, 84, 83
	93	75, 79, 81	81, 85, 87
Lotion	47	40, 41, 41	85, 87, 87
	70	57, 61, 62	81, 87, 89
	93	83, 86, 80	89, 92, 86

mine if a correlation existed between the levels of known *N*-nitrosamines and potential nitrosating agents. Results are shown in Table 2. In each of the products, potential nitrosating agents at levels ranging from 113 to 5021 ppb were found. There was no consistent correlation between levels of potential nitrosating agents and known *N*-nitrosamines; however, the highest levels of nitrosating agents were most often associated with the highest levels of *N*-nitrosamines. Several other factors are known to influence *N*-nitrosamine formation, including availability of secondary amines, pH, presence of antioxidants or other nitrite-scavenging compounds (2), and type of surfactant in an emulsion (24). These factors may explain the inconsistent correlation between levels of known *N*-nitrosamines and apparent nitrosating agents. In some products, there also may have been other unidentified *N*-nitrosamines present. Despite the lack of a consistent correlation, the results show the method could be used to screen cosmetic products that are likely to contain high levels of *N*-nitrosamines. Among 16 products analyzed in this study, 4 of the 5 products with levels of nitrosating agents in excess of 950 ppb had levels of *N*-nitrosamines greater than 3000 ppb.

Table 2. Potential nitrosating agents, NMPABAO[a], and NDELA[b] in cosmetic products

Sample No.	NMPABAO, ppb	NDELA, ppb	Nitrosating agents,[c] ppb
1[d]	3 000	390	267
2	ND[e]	270	113
3	ND	ND	953
4	ND	ND	213
5[d]	4 430	200	213
6[d]	4 240	120	240
7	160	—[f]	293
8	ND	—	260
9	350	—	193
10	ND	—	380
11	ND	—	267
12[d]	7 930	—	1 647
13	ND	—	280
14[d]	3 270	—	1 380
15[d]	7 200	—	5 021
16[d]	21 000	—	3 047

[a] 2-Ethylhexyl 4-(*N*-methyl-*N*-nitrosamino) benzoate.
[b] *N*-Nitrosodiethanolamine.
[c] Calculated as nitrite.
[d] Contained 2-bromo-2-nitro-1,3-propanediol (BNPD).
[e] ND = none detected.
[f] — = not analyzed because NDELA precursors were not present.

The method will be used in future surveys of cosmetic products as an indicator of the potential for the presence of *N*-nitrosamines. Because the method is rapid and selective and requires no sample preparation, numerous products can be screened rapidly, and only those with the highest levels of potential nitrosating agents will be evaluated further for *N*-nitrosamine contamination. Use of this screening procedure during product development might help avoid conditions that can lead to formation of *N*-nitrosamines in finished cosmetic products.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Reserpine and Rescinnamine in *Rauwolfia serpentina* Powders and Tablets: Collaborative Study

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A liquid chromatographic (LC) method for determining reserpine and rescinnamine in *Rauwolfia serpentina* powders and tablets, which uses fluorescence detection, was subjected to a collaborative study. The procedure for extraction and purification is a simplified version of that used in the current official method for analysis of these products. LC separations are performed on a normal-phase column. The mobile phase is methanol to which a small volume of an aqueous solution of 1-pentanesulfonic acid sodium salt can be added to achieve desired elution characteristics. Reserpine and rescinnamine elute at approximately the same time but can be individually quantitated by appropriate settings of the fluorescence detector. Reserpine is determined at an excitation wavelength of 280 nm and an emission wavelength of 360 nm, because rescinnamine is completely nonfluorescent at these wavelengths. Rescinnamine is determined at an excitation wavelength of 330 nm and an emission wavelength of 435 nm, because reserpine is completely nonfluorescent at these wavelengths. The following materials were used for the study: one sample of United States Pharmacopeia (USP) standard *R. serpentina* powder, one tablet type labeled as containing 100 mg *R. serpentina* and 2 tablet types labeled as containing 50 mg *R. serpentina*. For each of the 4 materials, 2 pairs of blind duplicates were prepared. Three materials were analyzed in duplicate by 8 laboratories. One of the 2 tablets labeled to contain 50 mg *R. serpentina* was analyzed only by 7 of 8 participating laboratories. Average combined content of reserpine and rescinnamine was 0.144% for the USP raw material and 0.132, 0.135, and 0.137% for the 3 commercial tablets. Reproducibility relative standard deviation values were 5.72, 5.93, 8.61, and 3.48% and repeatability relative standard deviation values were 2.57, 4.87, 3.19, and 1.99% for the 4 samples. The Associate Referee conducted a study to determine recoveries of reserpine plus rescinnamine by this method from mixtures simulating sample extracts. Average recovery of 15 determinations was 100.1%, with a relative standard deviation of 1.3%. The LC method for determination of reserpine and rescinnamine in *R. serpentina* powders and tablets has been adopted first action by AOAC INTERNATIONAL.

Preparations containing the ground root of *Rauwolfia serpentina* are used to treat hypertension and psychosis (1). *R. serpentina* contains at least 25 alkaloids, but its therapeutic properties are due primarily to reserpine and rescinnamine (1). AOAC has 2 official methods for analysis of these products, one of which (2) is practically identical to the United States Pharmacopeia (USP) method (3). The procedure that constitutes the basis of both methods (2, 3) was proposed by Banes et al. (4) and later subjected to a collaborative study (5). These methods measure the total amount of reserpine- and rescinnamine-group alkaloids and not the 2 compounds specifically or individually. Subsequently, a procedure was published based on column chromatography which permitted determination of reserpine and rescinnamine in *R. serpentina* (6). Results obtained by both methods (4, 6) showed that the combined reserpine and rescinnamine content represents about 85% of the total amount of reserpine- and rescinnamine-group alkaloids.

Another AOAC Official Method (7), adopted after a collaborative study by Smith (8), contains several modifications of the original. The intent is still to measure total amount of reserpine- and rescinnamine-group alkaloids, and results generally are identical to those of the other 2 earlier methods quoted (2, 3).

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The recommendation was approved by the Methods Committee on Drugs and Related Topics, and was adopted by the Official Methods Board of the Association. *See* "Official Methods Board Actions" (1997) *J. AOAC Int.* **80**, 84A, and "Official Methods Board Actions" (1997) *Inside Laboratory Management*, August issue.

A procedure for determining reserpine and rescinnamine in *R. serpentina* by liquid chromatography with fluorescence detection also has been reported (9). Modifications of the method were introduced, and the revised procedure was subjected to a collaborative study. The results of that study are reported in this paper.

Collaborative Study

Materials analyzed for this study were one sample of *Rauwolfia serpentina* USP powder (3) and 3 different samples of tablets labeled to contain *R. serpentina*. The USP powder was used as received. Two portions were made and each was assigned a unique random number.

For each of the 3 samples of tablets, two 60-tablet composites were prepared, and each one was assigned a unique random number. The average tablet weight was calculated for each composite, and the tablets were ground to pass a 0.25 mm mesh sieve. Portions of each prepared sample were placed in small plastic bags identified by sample number and sent to collaborators. Quantities supplied of each prepared sample were sufficient to perform at least 3 determinations. Collaborators were informed that samples could be stored at room temperature. Collaborators were instructed to perform one analysis of each sample. Remaining sample portions could be used to repeat a determination in case of a laboratory mishap. Each collaborator was supplied with a copy of the method and instructions, which indicated the average tablet weight of the ground composites and other necessary data.

997.10, Determination of Reserpine and Rescinnamine in *Rauwolfia serpentina*

First Action 1997

(Applicable to powders and commercial tablets.)
Method Performance:
See Table **997.10** for method performance data.

A. Principle

A portion of the commercial sample, expected to contain 100 mg *R. serpentina* is dispersed in 10.0 mL methanol and filtered. A 2.0 mL portion of the filtrate is purified by liquid–liquid partitioning. Chloroform extracts of the purified material are evaporated to dryness. The residue is dissolved in 25.0 mL methanol and analyzed for reserpine and rescinnamine by liquid chromatography (LC) with fluorescence detection.

B. Apparatus and Reagents

(a) *Reserpine.*—USP grade (United States Pharmacopeial Convention, Rockville, MD; Cat. No. 60100-00).

(b) *Rescinnamine.*—Sigma Chemical Co. (St. Louis, MO; Cat. No. R2127) or ICN K and K Laboratories (Costa Mesa, CA; Cat. No. 156504).

(c) *Chloroform.*—ACS grade.

(d) *Methanol.*—LC grade.

(e) *Sulfuric acid.*—ACS grade.

(f) *Filter paper.*—9.0 cm diameter, fast speed (No. 41, Whatman Lab. Div., Springfield Mill, Maidstone, KY, or equivalent).

(g) *Metal sieve.*—0.250 mm openings (Thomas Scientific Co., Swedesboro, NJ; Cat. No. 8323-M-48).

(h) *Water bath.*—Equipped with controls to maintain temperature at 60° ± 2°C.

(i) *1-Pentanesulfonic acid sodium salt.*—Sigma Chemical Co.; Cat. No. P-0299, or equivalent.

(j) *Injection valve.*—Equipped with 20 μL loop (Rheodyne, Inc., Cotati, CA, or equivalent).

(k) *LC column.*—Normal phase, 300 × 3.9 mm id (μPorasil; Millipore Corp., Bedford, MA, or equivalent).

(l) *Solvent delivery system.*—Millipore Corp., or equivalent.

(m) *Fluorescence LC detector.*—Shimadzu Corp., Kyoto, Japan, or equivalent.

(n) *Strip chart recorder.*—Or electronic integrator.

C. Solutions

Note: Solutions of reserpine and rescinnamine in methanol are stable for at least 2 weeks in the dark at room temperature.

(a) *Reserpine stock solution.*—Weigh accurately ca 50 mg reserpine and transfer to 100 mL volumetric flask. Add 1.0 mL chloroform, swirl to dissolve, and dilute to volume with methanol. Stopper and mix.

(b) *Rescinnamine stock solution.*—Weigh accurately ca 25 mg rescinnamine and transfer to 100 mL volumetric flask. Add 1.0 mL chloroform, swirl to dissolve, and dilute to volume with methanol. Stopper and mix.

(c) *Intermediate combined standard solution.*—Pipet 2.0 mL each of the 2 stock solutions into a 100 mL volumetric flask. Dilute to volume with methanol. Stopper and mix.

(d) *Combined standard solution.*—Pipet 8.0 mL intermediate solution **(c)** into a 100 mL volumetric flask. Dilute to volume with methanol, stopper, and mix.

(e) *Dilute sulfuric acid.*—Carefully add 14 mL sulfuric acid to 1000 mL water. Cool to room temperature.

(f) *Salt solution.*—Dissolve 1 g 1-pentanesulfonic acid sodium salt in 50 mL water.

D. Sample Preparation

Perform extractions and subsequent evaporation of extracts in suitable hood.

(a) *Rauwolfia serpentina powder.*—Weigh accurately ca 100 mg powder, transfer to 10 mL volumetric flask, and add 6 mL methanol. Stopper flask and shake vigorously for 2 min. Place in water bath *B*(**h**) for 10 min and swirl occasionally.

Table 997.10. Results of analysis of samples

Lab. No.	Replicate	RS, %[a]	RC, %[b]	(RS + RC), %	(RS + RC/RS), %
		Sample 1: USP powdered *Rauwolfia serpentina*[a]			
1	1	0.092	0.048	0.140	1.52
	2	0.093	0.050	0.143	1.54
2	1	0.084	0.048	0.132	1.57
	2	0.089	0.047	0.136	1.53
3	1	0.087	0.045	0.132	1.52
	2	0.094	0.046	0.140	1.49
4	1	0.098	0.053	0.151	1.54
	2	0.100	0.057	0.157	1.57
5	1	0.092	0.052	0.144	1.57
	2	0.095	0.050	0.145	1.53
6	1	0.089	0.061	0.150	1.69
	2	0.096	0.061	0.157	1.64
7	1	0.088	0.048	0.136	1.55
	2	0.092	0.050	0.142	1.54
8	1	0.098	0.053	0.151	1.54
	2	0.099	0.055	0.154	1.56
Average		0.093	0.051	0.144	1.56
RSD_R, %				5.72	
RSD_r, %				2.57	
		Sample 2: tablets labeled as containing 100 mg *Rauwolfia serpentina*[b]			
1	1	0.096	0.040	0.136	1.42
	2	0.094	0.036	0.130	1.38
2	1	0.094	0.036	0.130	1.38
	2	0.090	0.031	0.121	1.34
3	1	0.093	0.047	0.140	1.51
	2	0.095	0.033	0.128	1.35
4	1	0.093	0.040	0.133	1.43
	2	0.095	0.044	0.139	1.46
5	1	0.093	0.038	0.131	1.41
	2	0.091	0.036	0.127	1.40
6	1	0.090	0.037	0.127	1.41
	2	0.100	0.045	0.145	1.45
7	1	0.093	0.037	0.130	1.40
	2	0.081	0.034	0.115	1.42
8	1	0.102	0.039	0.141	1.38
	2	0.100	0.039	0.139	1.39
Average		0.094	0.038	0.132	1.41
RSD_R, %				5.93	
RSD_r, %				4.87	

Remove flask from bath, cool to room temperature, and dilute contents to volume with methanol. Stopper and mix. Filter solution, discarding first mililiter. Collect filtrate in a small glass-stoppered flask. Transfer 2.0 mL filtrate to 60 mL separator containing 20 mL dilute sulfuric acid, stopper, and mix. Extract with 10 mL chloroform. Filter extract through chloroform-washed cotton and collect in 50 mL glass-stoppered flask. Extract with 10 mL chloroform 4 additional times. Filter each portion and collect in flask.

After extractions, immediately begin evaporation of combined chloroform extracts, using water bath. A gentle stream of air may be used to assist evaporation. Remove flask from bath as soon as evaporation is complete and allow it to cool. Store at room temperature until ready for LC analysis. Pipet 25.0 mL methanol into flask, stopper, and shake well to disperse residue.

(b) *Rauwolfia serpentina tablets.*—Weigh 20 tablets and calculate average weight of a tablet. Grind tablets to pass 0.25 mm sieve and mix powder well. Weigh an amount of ground composite equivalent to ca 100 mg declared *R. serpentina* and transfer to 10 mL volumet-

Table 997.10. (*continued*)

Lab. No.	Replicate	RS, %[a]	RC, %[b]	(RS + RC), %	(RS + RC/RS), %
		Sample 3: tablets labeled as containing 50 mg *Rauwolfia serpentina*[c]			
1	1	0.092	0.036	0.128	1.39
	2	0.094	0.038	0.132	1.40
2	1	0.084	0.034	0.118	1.40
	2	0.086	0.031	0.117	1.36
3	1	0.090	0.031	0.121	1.34
	2	0.090	0.033	0.123	1.37
4	1	0.086	0.048	0.134	1.56
	2	0.108	0.051	0.159	1.47
5	1	0.098	0.041	0.139	1.42
	2	0.097	0.047	0.144	1.48
6	1	0.095	0.048	0.143	1.51
	2	0.092	0.056	0.148	1.61
7	1	0.099	0.036	0.135	1.36
	2	0.101	0.040	0.141	1.40
8	1	0.101	0.041	0.142	1.41
	2	0.100	0.041	0.141	1.41
Average		0.095	0.040	0.135	1.43
RSD_R, %				8.61	
RSD_r, %				3.19	
		Sample 4: tablets labeled as containing 50 mg *Rauwolfia serpentina*[c]			
1	1	0.134	0	0.134	
	2	0.138	0	0.138	
2	1	0.128	0	0.128	
	2	0.132	0	0.132	
3[d]	—	—	—	—	
4	1	0.137	0	0.137	
	2	0.134	0	0.134	
5	1	0.134	0	0.134	
	2	0.136	0	0.136	
6	1	0.146	0	0.146	
	2	0.137	0	0.137	
7	1	0.142	0	0.142	
	2	0.144	0	0.144	
8	1	0.138	0	0.138	
	2	0.135	0	0.135	
Average		0.137	0	0.137	
RSD_R, %				3.48	
RSD_r, %				1.99	

[a] RS, % = mg reserpine in 100 mg powder; RC, % = mg rescinnamine in 100 mg powder.

[b] RS, % = mg reserpine per tablet or in 100 mg declared *R. serpentina*; RC, % = mg rescinnamine per tablet or in 100 mg declared *R. serpentina*.

[c] RS, % = mg reserpine in 2 tablets or in 100 mg declared *R. serpentina*; RC, % = mg rescinnamine in 2 tablets or in 100 mg declared *R. serpentina*.

[d] Laboratory did not analyze sample.

ric flask. Continue as in (**a**) starting with ". . . and add 6 mL methanol . . ."

E. LC Determination of Reserpine

(**a**) *System suitability.*—Set detector at 280 nm excitation and 360 nm emission. Equilibrate column with methanol for 30 min at a flow rate of 1.0 mL/min. Continue column equilibration until retention times of peaks in 2 successive chromatograms of the combined standard solution *C*(**d**) do not differ by more than 0.2 min. Adjust flow so that peak appears after ca 8 min (Figure 1A). If a flow greater than 1.5 mL/min is required, prepare new mobile phase by mixing 0.5 mL salt solution *C*(**f**) with 1000 mL methanol. Reequilibrate column and similarly inject portions of the com-

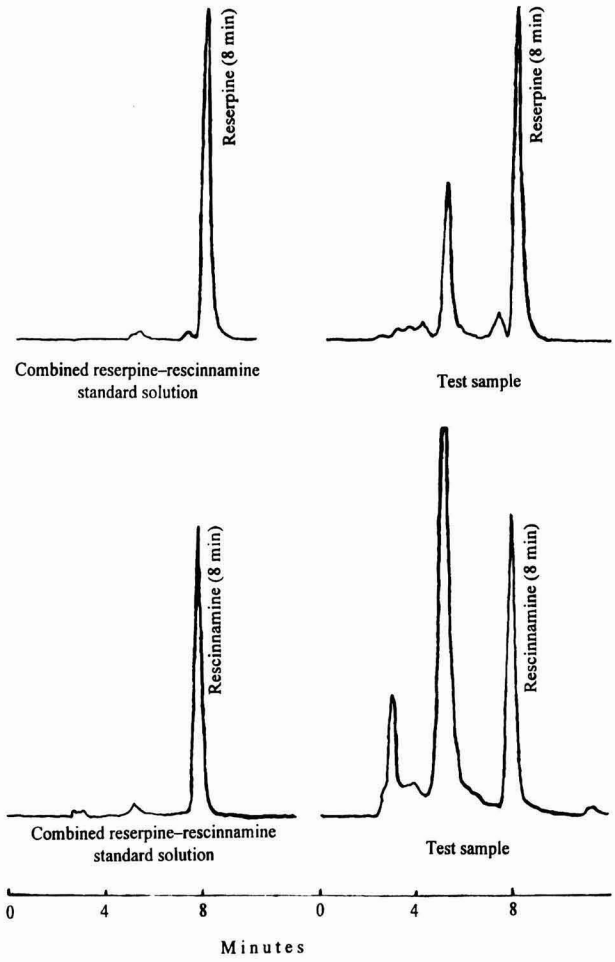

Figure 1. LC determinations with fluorescence detection: A, reserpine (excitation, 280 nm; emission, 360 nm); B, rescinnamine (excitation, 330 nm; emission 435 nm). Numbers indicating minutes apply to all chromatograms.

bined standard solution. If necessary, add small volumes of salt solution until peak elutes not later than 8 min at a flow not greater than 1.5 mL/min.

Typically, the volume of salt solution needed does not exceed 3 mL/1000 mL methanol. Adjust sensitivity so that the peak height is ca 60% of strip chart full scale. Tailing factor, T, calculated according to the formula in reference 10, must not be greater than 2.0. The relative standard deviation (RSD) of peak responses, by area or height, in 4 successive chromatograms must not be greater than 2.5%.

(**b**) *Procedure.*—Inject in succession one portion of the combined standard solution, 2 portions of the sample solution, and one additional portion of the combined standard solution.

(**c**) *Calculations.*—Calculate mean peak response from standard immediately preceding and following sample chromatograms and designate as R_s.

Average responses of corresponding peaks from the 2 sample chromatograms and designate as R_u.

Calculate reserpine found (RS) as follows:

$$\text{For powders, } RS, \% = R_u \times C \times 12\,500/R_s \times W$$

$$\text{For tablets, } RS, \% = R_u \times C \times 12\,500 \times T/R_s \times W \times L$$

where C = concentration (mg/mL) reserpine in combined standard solution; W = weight of sample (mg); T = average weight of a tablet (mg); L = labeled amount of *R. serpentina* (mg/tablet).

F. LC Determination of Rescinnamine

Proceed as in *E. LC Determination of Reserpine*, but set excitation of fluorescence detector at 330 nm

and emission at 435 nm. Peak elutes after 8 min (Figure 1B).

Note: Sample chromatograms have additional very late eluting peaks that are detected only when the detector is set for the determination of rescinnamine.

If previous injections of sample solutions were made, the column must be washed for at least 4 h at a flow of 1 mL/min before starting determinations of rescinnamine. With a properly washed column, late peaks should not begin to appear until after 3 h of continued analysis.

Average peak responses from standard chromatogram preceding and following the 2 sample chromatograms and designate as R_s1. Average responses of corresponding peaks in 2 sample chromatograms and designate as R_u1.

Calculate rescinnamine found (RC) as follows:

$$\text{For powders, } RC, \% = R_u1 \times C1 \times 12\,500/R_s1 \times W$$

$$\text{For tablets, } RC, \% = R_u1 \times C1 \times 12\,500 \times T/R_s1 \times W \times L$$

where $C1$ = concentration (mg/mL) of rescinnamine in combined standard solution.

Ref.: *J. AOAC Int.* **81**, 373 (1998).

Results and Discussion

The first step of the method consists of dispersing sample in methanol. Vigorous shaking followed by heating is necessary to ensure complete extraction of alkaloids from the powder. The next step is a purification process that isolates reserpine and rescinnamine from most of the other alkaloids in the methanolic extract. Recoveries reported in Table 1 were determined by the Associate Referee and reflect only the purification process. It is very difficult or impossible to test for completeness of extraction in the initial step, primarily because quantities of the alkaloids in the samples already are so small. For the recovery study, 3 different methanolic solutions were prepared, containing quantities of the 2 alkaloids within the range normally present in the methanolic extracts of the samples. Five 2.0 mL portions from each of the 3 solutions were subjected to the same purification procedure applied to sample extracts. Quantities of the 2 alkaloids were determined by the method described. Average recovery of reserpine was 100.4% (RSD, 1.2%) and that of rescinnamine was 99.2% (RSD, 2.6%). The better reproducibility of the reserpine determinations is due to its higher fluorescence intensity compared with that of rescinnamine. When the totals of the 2 alkaloids are considered, average recovery is 100.1% and RSD is 1.3%.

Eight laboratories participated in the study but one of them, for lack of time, analyzed only 3 of the 4 pairs of blind duplicates. None of the collaborators reported any difficulties with the extraction and purification procedure. Equilibration of the column required different amounts of time in each laboratory. In some cases, times as long as 4 or 5 h were needed. Equilibration times were higher if the column had been previously used with a mobile phase considerably different from the one used in this method. Time required for equilibration was, however, much shorter in successive reserpine and rescinnamine runs.

Table **997.10** reports results obtained by the collaborators. For sample 1, *R. serpentina* USP powder, average amount of reserpine found was 0.093 mg/100 mg powder or 0.093%; average amount of rescinnamine found was 0.051 mg/100 mg powder or 0.051%. Combined reserpine and rescinnamine content of the powder was 0.144%.

For sample 2, tablets labeled as containing 100 mg *R. serpentina*, average reserpine content was

Table 1. Recovery of reserpine (RS) and rescinnamine (RC) by the purification procedure of the method[a]

Solution No.	Determination No.	Recovery, %		
		RS	RC	RS + RC
1	1	98.6	101.5	99.6
	2	101.0	102.5	101.5
	3	99.4	97.0	98.6
	4	100.4	99.7	100.2
	5	100.5	99.2	100.1
2	1	99.5	98.4	99.1
	2	100.5	103.9	101.6
	3	100.8	101.4	101.0
	4	99.8	100.5	100.0
	5	99.5	96.6	98.5
3	1	103.6	100.2	102.5
	2	99.8	96.3	98.6
	3	102.2	99.5	101.3
	4	100.2	95.1	98.5
	5	100.2	96.2	99.9
Average		100.4	99.2	100.1
RSD, %		1.2	2.6	1.3

[a] Three solutions were prepared in which the concentrations of the 2 ingredients were as follows:

Solution No.	Concentration, mg/mL		
	RS	RC	RS + RC
1	0.0100	0.0050	0.0150
2	0.0090	0.0045	0.0135
3	0.0110	0.0055	0.0165

A 2.0 mL aliquot of a solution was transferred to a separator containing 20 mL dilute sulfuric acid. The same procedure used for sample analysis was then followed. Five determinations were made for each of the 3 solutions.

0.094 mg/tablet or 0.094% of the labeled amount of *R. serpentina*; the average rescinnamine content was 0.038 mg/tablet or 0.038% of the labeled amount of *R. serpentina*. The sum of the 2 alkaloids represents 0.132% of the labeled amount of *R. serpentina*.

For samples 3 and 4, tablets labeled as containing 50 mg *R. serpentina*, amount of sample required for analysis and containing 100 mg declared *R. serpentina* is equivalent to the average weight of 2 tablets. Average reserpine content found was 0.095 mg per 2 tablets or in 100 mg declared *R. serpentina*; average rescinnamine content was 0.040 mg per 2 tablets or in 100 mg declared *R. serpentina*. The sum of the 2 alkaloids represents 0.135% of labeled amount. The reserpine content in these 2 samples is very close to that found in 100 mg *R. serpentina* but that of rescinnamine is lower, the most likely explanation being that the material used for the formulation contained a proportionately lower amount of this alkaloid. It is also possible that rescinnamine is more difficult to extract in presence of tablet excipients.

For sample 4, the average reserpine content found was 0.137 mg per 2 tablets or 0.137% of labeled amount of *R. serpentina*, but no rescinnamine was found. The absence of rescinnamine indicates that the manufacturer used another type of *Rauwolfia* material instead of *R. serpentina*. Banes et al. (6) list some raw materials containing reserpine but not rescinnamine. Because the 2 alkaloids have identical therapeutic properties, the efficacy of a commercial product is determined by total content rather than by individual contents. The ratio of the contents however may be useful for identification. For this reason, ratios were calculated by the Associate Referee and also reported in Table 2. Statistical evaluation of data was limited to total content of the 2 alkaloids, because this result is the most significant; all data received were used. Reproducibility relative standard deviation (RSD_R) values for the 4 samples, in the order listed previously, were 5.72, 5.93, 8.61, and 3.48%. The RSD_R value for sample 2, tablets declaring 100 mg *R. serpentina*, was very close to that of the raw material. The highest RSD_R value was obtained for sample 3, tablets declaring 50 mg *R. serpentina*. This was probably because in this particular sample the content of tablet excipients was unusually high. The lowest RSD_R value was obtained for sample 4, tablets also declaring 50 mg *R. serpentina*. The better reproducibility in this case was due in part to the fact that the average tablet weight was less than for sample 3, but primarily to the absence of rescinnamine, the determination of which, as previously indicated, is less reproducible than that of reserpine. Repeatability standard deviation (RSD_r) values were 2.57, 4.87, 3.19, and 1.99%, respectively.

Table 2. Comparison of results with those of a current AOAC method

Sample[a]	Method 1[b] (RS + RC), %	Method 2[c] AOAC 20.9.05	Ratio of results method 1/method 2
1	0.144	0.170	0.847
2	0.132	0.159	0.830
3	0.135	0.162	0.833
4	0.137	0.160	0.856

[a] Refer to Table **997.10** for identification of samples.

[b] Average results as reported in Table **997.10**.

[c] Average of 2 determinations on each sample made by the Associate Referee.

Table 2 compares combined reserpine and rescinnamine contents obtained by this method with total contents of reserpine- and rescinnamine-group alkaloids as determined by one of the AOAC official methods (7). The combined reserpine and rescinnamine content represented approximately 85% of reserpine- and rescinnamine-group alkaloids. The ratio agrees with the estimation previously reported by Banes et al. (6), using different methods. The related alkaloid primarily responsible for the higher results by any of the current official methods (2, 3, 7) was identified in a previous investigation (11) as raubasinine (reserpinine). This compound reacts similarly in the methods quoted (2, 3, 7) but does not have the therapeutic properties of reserpine and rescinnamine. Raubasinine is only partially extracted by this method, and in the LC analysis, it elutes earlier than reserpine, as can be seen from chromatogram B of Figure 1, where raubasinine appears after about 4 min.

The USP specification for powdered *R. serpentina* (3) is that it should contain between 0.15 and 0.20% of reserpine- and rescinnamine-group alkaloids. Similarly, tablets are required to contain an amount of reserpine- and rescinnamine-group alkaloids between 0.15 and 0.20% of the declared amount of *R. serpentina*.

The method collaboratively studied for this report determines only reserpine and rescinnamine. Consequently, if this method is used for regulatory purposes, specifications have to be adjusted. After a careful examination of the collaborative study results, the Associate Referee recommends the following limits for *R. serpentina* products analyzed by this method: For powdered *R. serpentina*, the combined reserpine and rescinnamine content should be between 0.13 and 0.16%. For tablets, the combined content of reserpine and rescinnamine should be between 0.12 and 0.16% of the labeled amount of *R. serpentina*. The limit is lower for tablets to account for greater difficulties in initial extraction with methanol.

A significant difference between this method and the one in the USP (3) is sample size. In this method, the sample weight required for an analysis contains about 100 mg *R. serpentina*. The USP (3) requires a sample weight containing 2.5 g (2500 mg) *R. serpentina*. With larger samples, results may be more uniform, but a method like the one in the USP (3) is not too suitable for determining uniformity of dosage. With appropriate

modifications, the method presented here also could be used to determine reserpine and rescinnamine in individual tablets.

Recommendations

The Associate Referee recommends that the LC method for determining reserpine and rescinnamine in *R. serpentina* products be adopted as official first action and that limits be set as follows: For powdered *R. serpentina*, the combined content of reserpine and rescinnamine should be between 0.13 and 0.16%. For tablets, the combined content of reserpine and rescinnamine should be between 0.12 and 0.16% of the labeled amount of *R. serpentina*.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Simultaneous Liquid Chromatographic Analysis of the β-Lactam Antibiotics Cefazolin, Cefadroxil, Cephalexin, Ampicillin, and Cephradine in Solution

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A liquid chromatographic method was developed for the determination of nanogram quantities of 5 broad-spectrum structurally related β-lactam antibiotics (cefazolin, cefadroxil, cephalexin, cephradine, and ampicillin) in solution. The method uses a C_{18} reversed-phase column, UV absorption (240 nm) detection, and an aqueous mobile phase containing isopropyl alcohol and acetic acid. Relative resolution between the antibiotic peaks ranged from 1.7 to 5.9 for all peaks. Chromatographic retention times were 2.97, 3.92, 4.57, 5.37, and 6.56 min for cefazolin, cefadroxil, cephalexin, ampicillin, and cephradine, respectively. Accuracy, precision, linearity, and long term analytical reproducibility were determined by statistical analysis. Use of the proposed method to evaluate the degradation of cephradine solutions stored at room temperature illustrated its potential as a stability-indicating assay.

Cefazolin, cefadroxil, cephalexin, ampicillin, and cephradine (Figure 1) are semi-synthetic cephalosporin β-lactam antibiotics that are active against both Gram-positive and Gram-negative bacteria (1) and are widely used for the treatment of infections. However, the in vitro bactericidal activity of an antibiotic does not always correlate with its therapeutic efficacy for many reasons (2). In recent years, a growing market demand (1) has served to intensify synthesis efforts on the part of pharmaceutical companies, leading to the introduction and subsequent approval of a variety of β-lactams with higher potency and/or broader spectral range. Methods for the analysis of β-lactams include microbial assay (3), hydroxylamine assay (3), iodometric assay (1), immunoassay (4), nonaqueous titration for the acidic and basic functional groups (1), and liquid chromatography (LC; 1,5). Of all these methods, LC has proven to be superior to the others in its specificity, stability-indicating ability, and simultaneous analysis (1).

The purpose of this investigation was to develop an accurate, reproducible, and rugged LC assay to determine the presence of cefazolin, cefadroxil, cephalexin, ampicillin, and cephradine combined in solution and to evaluate the method for its use in assessing the stability of admixture solutions containing these β-lactams. Although published methods are available for some of these antibiotics, the present method is more convenient and efficient for the assay of large numbers of samples.

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Experimental

Apparatus

(a) *Columns.*—C_{18} reversed-phase 250 × 3.2 mm, 3 μm chromosphere (Phenomenex, Torrance, CA), C_{18} reversed-phase 250 × 4.62 mm, 5 μm chromosphere (Phenomenex), and C_{18} reversed-phase 250 × 4.6 mm, 5 μm ODS (Beckman Instruments, Fullerton, CA).

(b) *Liquid chromatograph.*—UV/Vis spectrophotometer multiple wavelength detector (Hewlett-Packard, ChemStation 1050, Palo Alto, CA) solvent delivery system, autosampler injector, and data station.

(c) *Chromatographic parameters.*—Flow rate, 1 mL/min; detector wavelength, 240 nm. Mobile phase, 10% acetic acid solution in water–isopropyl alcohol–water (4 + 9 + 87).

Reagents

(a) *Antibiotics.*—Cephradine hydrate, cephalexin, cefazolin sodium salt, and cefadroxil (Sigma Chemical Co., St. Louis, MO). Ampicillin sodium, USP Reference Standard (U.S. Pharmacopeial Convention, Rockville, MD).

(b) *Isopropyl alcohol.*—Glass-distilled, suitable for LC (EM Science, Gibbstone, NJ).

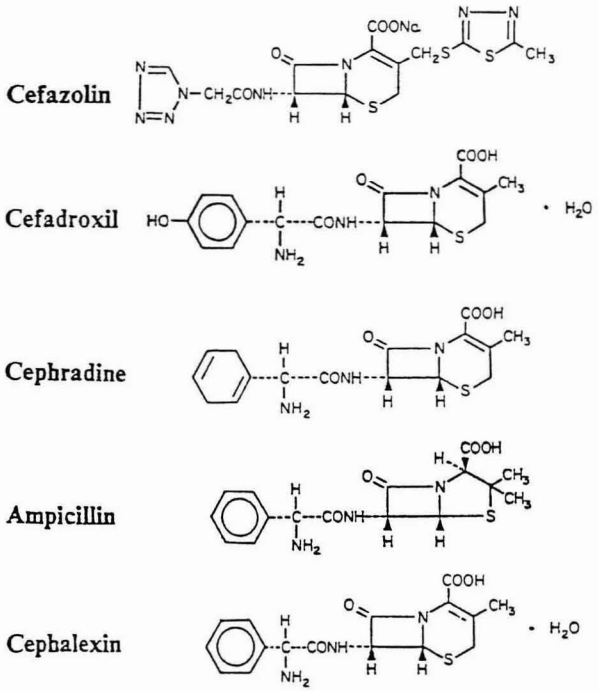

Figure 1. Chemical structures of ampicillin, cefazolin, cephradine, cephalexin, and cefadroxil.

(c) *Water.*—Milli-Q grade (MQ water, Waters Associates, Inc., Milford, MA).

Sample Preparation

A 20 mg portion of each antibiotic was weighed and dissolved quantitatively in 200 mL deionized water (these stock solutions can be stored at 0°–5°C for 5 days). Immediately before the analyses, working solutions were prepared by diluting each stock solution (1 + 9) with a refrigerated solution of 0.5% sodium chloride in MQ water (0.01 mg antibiotic/mL, or 10 μg/mL). The working solutions were prepared fresh every day and stored under refrigeration. The working admixture solution was prepared by carefully mixing 1 mL of each antibiotic stock solution and diluting the resultant solution (5 + 5) with 1% sodium chloride solution in MQ water. Immediately before starting the analyses, ca 2 mL of each antibiotic working solution and the admixture working solution were transferred to separate autosampler vials. The working solutions were diluted further for linearity and minimum quantitative studies.

LC Determination

The Chemstation was programmed to make one 5 μL injection of each of 11 vials. Each vial contained independently prepared samples of each antibiotic and the mixture. The precision of the assay was determined for each antibiotic. The same analysis was repeated once each week, for 7 weeks, with different concentrations each week (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, and 17.6 μg) to determine the week-to-week variation and the linearity of the method. To evaluate the short-term stability of reconstituted antibiotic solutions, the contents of each auto injector vial were allowed to stand at room temperature (25°C) for 3 days. Each solution was reassayed twice daily during this period.

Results

Acceptable separation was obtained between each pair of the 5 β-lactams as shown by the retention times and resolution in Figure 2. The relative standard deviations (RSD) of the retention times, peak areas, and peak heights are shown in Tables 1–3. The method was repeated with 3 different analytical colums and at different weeks. Table 4 shows that the method offers reasonable precision and is rugged. The method is stability-indicating for cephradine (Figure 3) and is linear in the range of 2.5–17.6 μg/mL. The linear correlation coefficients are shown in Table 4. The minimum

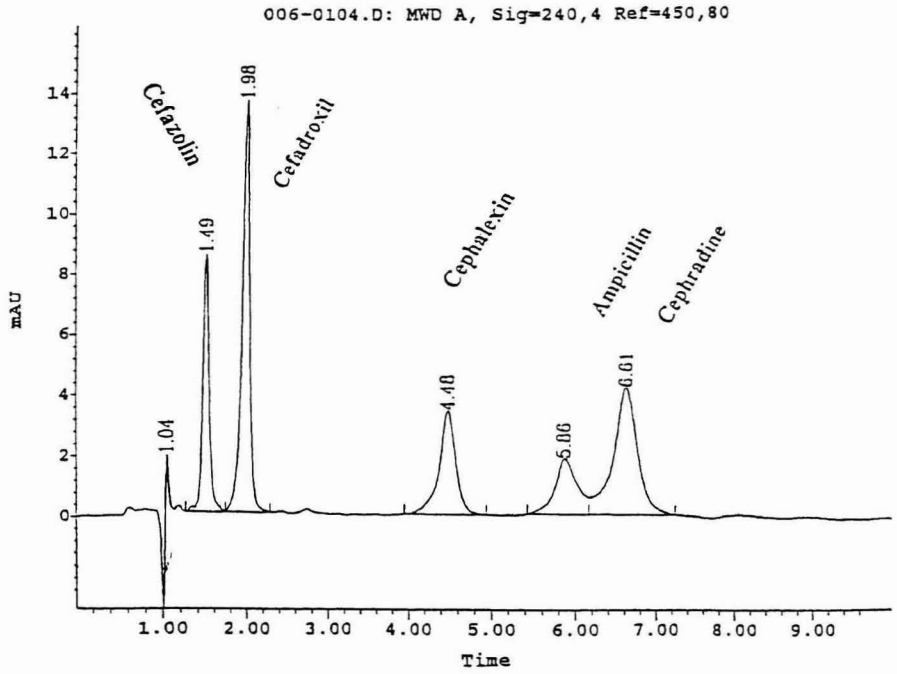

Figure 2. Separation of cefazolin, cefadroxil, cephalexin, ampicillin, and cephradine by LC method (see text for conditions).

Table 1. Precision of assay of cefazolin, cefadroxil, cephalexin, ampicillin, and cephradine (n = 11) by retention time, peak area, and peak height for 5 μL injection of 2.5 μg/mL solution

	Retention time			Mean peak response			
			Resolution		Area		Height
Compound	Min	RSD, %	(R)[a]	Area[b]	RSD, %	Height	RSD, %
Cefazolin	2.97	1.6		889	0.4	209	1.62
Cefadroxil	3.92	1.6	5.9	391	1.2	45	1.68
Cephalexin	4.57	1.4	2.8	480	1.1	53	0.00
Ampicillin	5.37	1.8	2.8	143	1.7	12	0.00
Cephradine	6.05	1.6	1.7	300	1.4	25	1.69

[a] Relative separation factors between each peak and its previous eluting peak.

[b] These data are also recorded in Table 4, column 1.

Table 2. Precision of assay of cefazolin, cefadroxil, cephalexin, ampicillin, and cephradine (n = 8) by retention time, peak area, and peak height for 5 μL injection of 5 μg/mL solution

	Retention time			Mean peak response			
			Resolution		Area		Height
Compound	Min	RSD, %	(R)[a]	Area[b]	RSD, %	Height	RSD, %
Cefazolin	2.88	0.6		1725	0.9	384	1.1
Cefadroxil	3.89	1.9	4.5	763	1.6	84	1.1
Cephalexin	4.49	1.0	2.8	931	1.8	92	1.1
Ampicillin	5.34	1.0	2.4	276	1.5	20	1.6
Cephradine	6.03	1.1	1.8	590	1.5	43	1.6

[a] Relative separation factors between each peak and its previous eluting peak.

[b] These data are also recorded in Table 4, column 2.

Table 3. Precision of assay of cefazolin, cefadroxil, cephalexin, ampicillin, and cephradine (n = 8) by retention time, peak area and peak height for 5 μL injection of 15 μg/mL solution

	Retention time			Mean peak response			
			Resolution		Area		Height
Compound	Min	RSD, %	(R)[a]	Area[b]	RSD, %	Height	RSD, %
Cefazolin	2.90	0.5		5353	1.4	805	1.5
Cefadroxil	3.93	0.6	4.3	2386	1.9	210	1.5
Cephalexin	4.50	0.5	2.4	2838	1.6	202	0.5
Ampicillin	5.33	0.5	2.2	838	1.5	43	1.3
Cephradine	6.05	0.7	1.4	1771	1.9	92	1.8

[a] Relative separation factors between each peak and its previous eluting peak.
[b] These data are also recorded in Table 4, column 6.

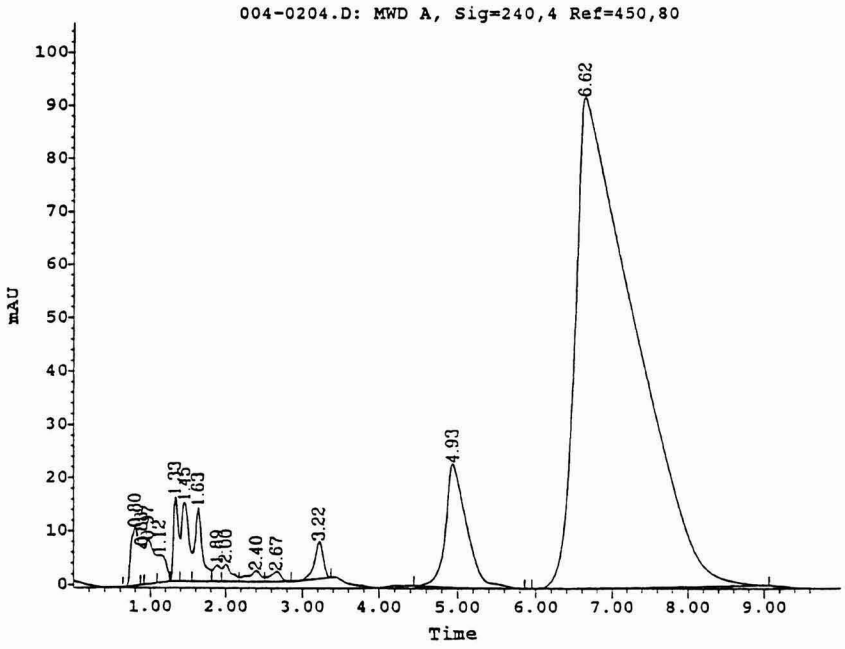

Figure 3. Chromatograms of cephradine standard (see text for conditions). This standard contains several measurable contaminants, demonstrating that the method is stability-indicating for cephradine.

Table 4. Calibration curve linearity of each antibiotic studied

Compound concentration (μg/mL)	Mean peak area response							
	2.5[a]	5.0[b]	7.5	10.0	12.0	15.0[c]	17.6	
Run time	at start	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	Linear correlation coefficient
Cefazolin	889	1725	2719	3635	4460	5352	6264	0.9998
Cefadroxil	391	763	1159	1613	2013	2386	2807	0.9997
Cephalexin	480	931	1398	1881	2394	2838	3392	0.9998
Ampicillin	143	276	408	545	719	838	1036	0.9984
Cephradine	300	590	890	1164	1491	1771	2152	0.9994

[a] These data are also presented in Table 1.
[b] These data are also recorded in Table 2.
[c] These data are also recorded in Table 3.

Table 5. Stability of cefazolin, cefadroxil, cephalexin, ampicillin, and cephradine solutions at refrigeration and room temperatures

	Percentage of assay after days refrigerated					Percentage of assay after days at room temperature		
Solution	1	2	3	4	5	1	2	3
Cefazolin,	100.0	100.5	99.0	99.3	101.7	100.0	95.4	94.2
Cefadroxil	100.0	99.4	101.9	100.8	98.1	100.0	96.6	90.9
Cephalexin	100.0	100.0	101.8	99.3	98.6	100.0	94.7	93.0
Ampicillin	100.0	99.1	101.5	99.8	101.1	100.0	95.5	92.8
Cephradine	100.0	100.5	99.4	99.0	98.8	100.0	88.7	62.1

levels of quantitation (10 to 1 signal-to-noise ratio) are cefazolin, 50 ng/mL; cefadroxil, 20 ng/mL; cephalexin, 40 ng/mL; ampicillin, 10 ng/mL; and cephradine, 20 ng/mL.

Short-term stability studies of the reconstituted antibiotic solutions showed that cephradine solution lost more than 38% potency during 3 days of storage at room temperature (Table 5). The other antibiotic solutions lost from 6 to 9% potency under similar storage conditions.

Conclusion

The proposed method provides a fast, accurate, and rugged assay with stability-indicating potential for these β-lactams in solution alone or in mixtures. It can also be used for a large number of samples.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Stability-Indicating Spectrophotometric and Densitometric Methods for Determination of Some Cephalosporins

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Two methods were developed for determination of intact ceftazidime (I), cefuroxime sodium (II), and cefotaxime sodium (III) in the presence of their degradation products. In the first method, first derivative spectrophotometry (D_1) is used. The (D_1) absorbance is measured at 268.6, 306, and 228.6 nm for I, II, and III, respectively. The first proposed method determines I, II, and III in concentration ranges of 5–50, 5–35, and 5–40 μg/mL, respectively, with corresponding mean accuracies of 99.7 ± 0.8, 100.1 ± 0.7, and 99.8 ± 0.8%. The method determines the intact drug in the presence of up to 90% degradation products for I, and II and up to 80% for III. The second method depends on the quantitative densitometric evaluation of thin-layer chromatograms of I, II, and III. It determines I, II, and III in concentration ranges of 4–16 μg for I and 2–12 μg for II and III, with mean accuracy's of 99.5 ± 0.8, 99.2 ± 0.7, and 99.7 ± 0.8% for I, II, and III, respectively. The second method retains its accuracy in the presence of up to 90% degradation products for the 3 drugs. The results obtained by applying the proposed methods were statistically analyzed and compared with those obtained by the official method.

Ceftazidime (I), cefuroxime sodium (II), and cefotaxime sodium (III) are semisynthetic beta-lactam antibiotics of the cephalosporin group. Their structures are shown in Figure 1.

Several methods have been reported for the determination of I, II, and III, including those using liquid chromatography (LC; 1–4), colorimetry (5–7), spectrophotometry (8), derivative spectrophotometry (9), fluorimetry (10), polarography (11, 12), and nuclear magnetic resonance spectrometry (13).

The purpose of this study was to determine the 3 drugs in the presence of their degradation products by simple, rapid, and selective stability-indicating assays for quality control and routine analysis.

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Experimental

Apparatus

(a) *UV/Vis spectrophotometer.*—Shimadzu 1601 PC attached to IBM computer compatible with UVPC personal spectroscopy, S/N UH3-0293 computer program, and Hewlett-Packard printer for Windows 6.1 Desk Jet 600 series version, software version 3.7.

(b) *Densitometer.*—Dual wavelength Shimadzu flying CS-9000 with video display and high-speed, high-quality, parallel-head printer/plotter.

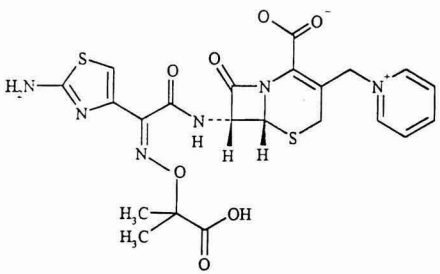
(c) *Thin-layer chromatography (TLC) plates.*—Precoated with Silica Gel GF, 0.25 mm thickness, fluorescent at 254 nm (E. Merck, Darmstadt, Germany).

Reagents

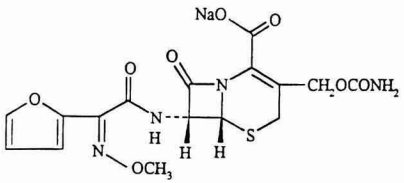
(a) *Pure substances.*—(*1*) *Ceftazidime.*—Kindly supplied by Glaxo Egypt. The purity was found to be 100.3 ± 0.6% by the United States Pharmacopeia (USP) method (14). (*2*) *Cefuroxime sodium.*—Kindly supplied by Glaxo Egypt (Cairo, Egypt). The purity was found to be 99.9 ± 0.3% by the USP method (14). (*3*) *Cefotaxime sodium.*—Kindly supplied by Hoechst Egypt (Cairo, Egypt). The purity of the sample was found to be 100.5 ± 0.5% according to the USP method (14).

(b) *Pharmaceutical formulations.*—(*1*) *Fortam for injection.*—Glaxo Batch No. 70806 A. Label claim for each vial was 1 g ceftazidime. (*2*) *Zinnat capsules.*—Glaxo Batch No. 70007 A. Label claim for each vial was 0.25 g cefuroxime. (*3*) *Zinnat suspension.*—Glaxo Batch No. 51814 A. Label claim for each 5 mL was 125 mg cefuroxime sodium. (*4*) *Claforan for injection.*—Hoechst Batch Nos. 008, 003 N, and 0.3 N. Label claim for each vial was 0.25, 0.5, or 1 g cefotaxime sodium, respectively.

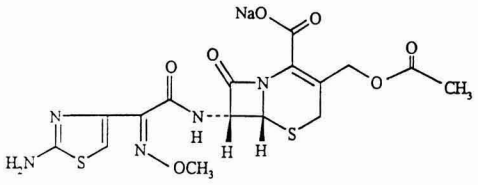
(c) *Chemicals.*—All chemicals were analytical grade and all solvents were spectroscopic grade. (*1*) *Metha-*



I. Ceftazidime



II. Cefuroxime Sodium



III. Cefotaxime Sodium

Figure 1. Structures of ceftazidime, cefuroxime sodium, and cefotaxime sodium.

nol.—E. Merck. (*2*) *Mobile phase for II and III.*—Methanol-concentrated ammonium hydroxide (100 + 1.5, v/v). (*3*) *Mobile phase for I.*—Methanol–1N acetic acid (25 + 75, v/v). (*4*) *Sulfuric acid.*—0.01N, aqueous solution. (*5*) *Sodium hydroxide.*—0.01N, aqueous solution.

(**d**) *Standard stock solutions.*—(*1*) *Ceftazidime.*—0.1 mg/mL methanol and 1 mg/mL methanol. (*2*) *Cefuroxime sodium.*—0.1 mg/mL water and 1 mg/mL methanol. (*3*) *Cefotaxime sodium.*—0.1 mg/mL water and 1 mg/mL methanol.

Preparation of Degradation Products

The degradation products of I, III, and III were laboratory-prepared according to the following method. In separate 100 mL flasks, 50 mL 0.01N sulfuric acid was added to 50 mg each of I, II, and III, and the flasks were heated in an oven at 105°C for 4 h. The solutions were cooled, neutralized with 0.1N sodium hydroxide, concentrated to ca 2 mL, transferred to 10 mL volumetric flasks, and diluted to volume with methanol. A 50 μL aliquot of each solution was applied in a band on a TLC plate; 10 μL of the standard methanolic solution of the corresponding drug (1 mg/mL) was also spotted as a reference. The plates were developed to 16 cm with methanol–1N acetic acid (25 + 75, v/v) as the mobile phase for I and with methanol-concentrated ammonium hydroxide (100 + 1.5, v/v) as the mobile phase for II and III; the plates were then removed and air-dried. The bands corresponding to the degradation products were visualized under UV light at 254 nm, scraped, and extracted, each with three 20 mL portions of methanol. The extracts were filtered and evaporated just to dryness on a boiling water bath. The residues left after evaporation were used for the laboratory-prepared mixtures.

Spectrophotometric Method

(**a**) *Construction of calibration curves.*—Accurately transfer aliquots of stock solutions (0.1 mg/mL) equivalent to 50–500 μg I, 50–350 μg II, and 50–400 μg III to separate 10 mL volumetric flasks. Dilute to volume with methanol for I and with water for II and III. Record the first derivative curve for each solution and measure the absorbance (D_1) at 268.6 nm for I, 306 nm for II, and 228.6 nm for III.

(**b**) *Assay of pharmaceutical formulations.*—(*1*) *Injections.*—Allow a container of the injection to dissolve, and mix the solution. Transfer an accurately measured volume of the injection, equivalent to ca 50 mg of the drug, to a 50 mL volumetric flask, dilute to volume with methanol for I and with water for III, and mix (1 mg/mL). Dilute an aliquot of each solution with methanol or water for I and III, respectively, to obtain a concentration of ca 0.1 mg/mL for each drug. Transfer 2 mL of each solution to a 10 mL volumetric flask. Proceed as described in *Construction of calibration curves*, starting with "Dilute to volume with methanol for I ...." (*2*) *Tablets.*—Accurately weigh 10 tablets and grind into a fine powder. Transfer powder equivalent to 50 mg II to a 50 mL volumetric flask. Dissolve powder in water and dilute to volume. Thoroughly mix contents of flask and filter. Proceed as described for injections, starting with "Dilute an aliquot of each solution ...." (*3*) *Oral suspensions.*—Constitute as directed in the labeling and filter. Wash the residue 3 times with water, and dilute the filtrate quantitatively to obtain a solution containing ca 0.1 mg/mL. Proceed as described for injections, starting with "Transfer 2 mL of each solution ...."

Densitometric Method

(**a**) *Construction of calibration curves.*—Accurately transfer aliquots of stock solutions (1 mg/mL) equivalent to 0.5–4.5 mg of each drug to separate 5 mL volumetric flasks, and dilute to volume with methanol. Apply 20 μL of each solution to a TLC plate (20 ×

20 cm), using a 20 μL pipet. Develop the plate to 16 cm, using methanol-concentrated ammonium hydroxide (100 + 1.5, v/v) for I and methanol–1N acetic acid (25 + 75, v/v) for II and III. Determine the spots densitometrically at 258, 272, and 262 nm for I, II, and III, respectively. Plot the calibration curves representing the relation between the recorded area under the peak and the corresponding concentration.

(b) *Assay of pharmaceutical formulations.*—Proceed as described in *Spectrophotometric Method, Assay of pharmaceutical formulations*, using 1 mg/mL solutions of the dosage forms. Transfer 2 mL of each solution into a separate 5 mL volumetric flask, and dilute to volume with methanol. Proceed as described in the previous section, *Construction of calibration curves*, starting with "Apply 20 μL of each solution ...."

Results and Discussion

Cephalosporins undergo a variety of hydrolytic degradation reactions which lead to many problems in their determination during quality control assays.

The most active site in cephalosporins is the 3-acetoxylmethyl group. In addition to its reactivity in nucleophilic displacement reactions, the acetoxyl function of this group also readily undergoes solvolysis in strong acidic solution to form the desacetyl cephalosporin derivatives. The latter lactonize to form the desacetyl cephalosporin lactones which are virtually inactive (15) (Figure 2). The degradation takes place in 3 steps (16).

For preparation of the degradation products, acid degradation was used. The degradation products were separated by TLC and LC (14). With LC, 3 distinct peaks other than that of the intact drug were obtained (Figure 3), whereas 1 spot was obtained by TLC. The TLC spot was scraped and used as a mixture of degradation products.

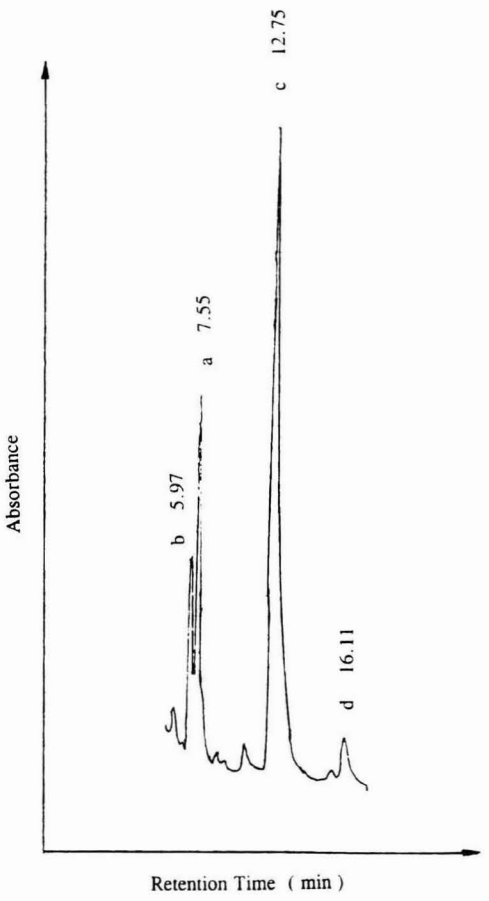


Figure 3. Liquid chromatograms obtained after acid hydrolysis of ceftazidime (a) produced the degradation products (b–d).

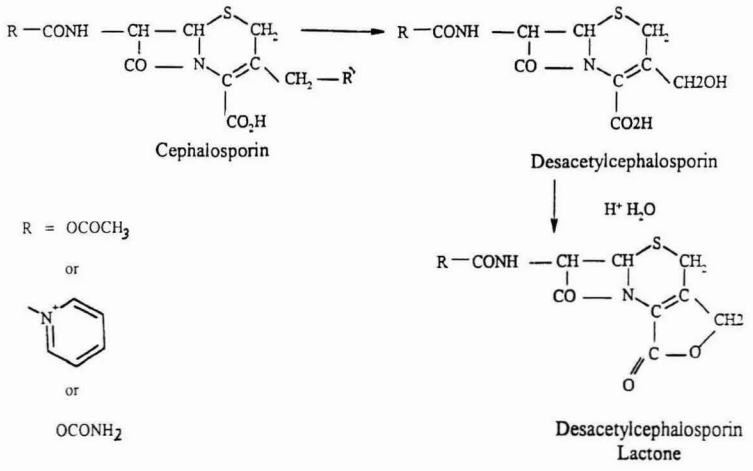


Figure 2. Degradation of the cephalosporins.

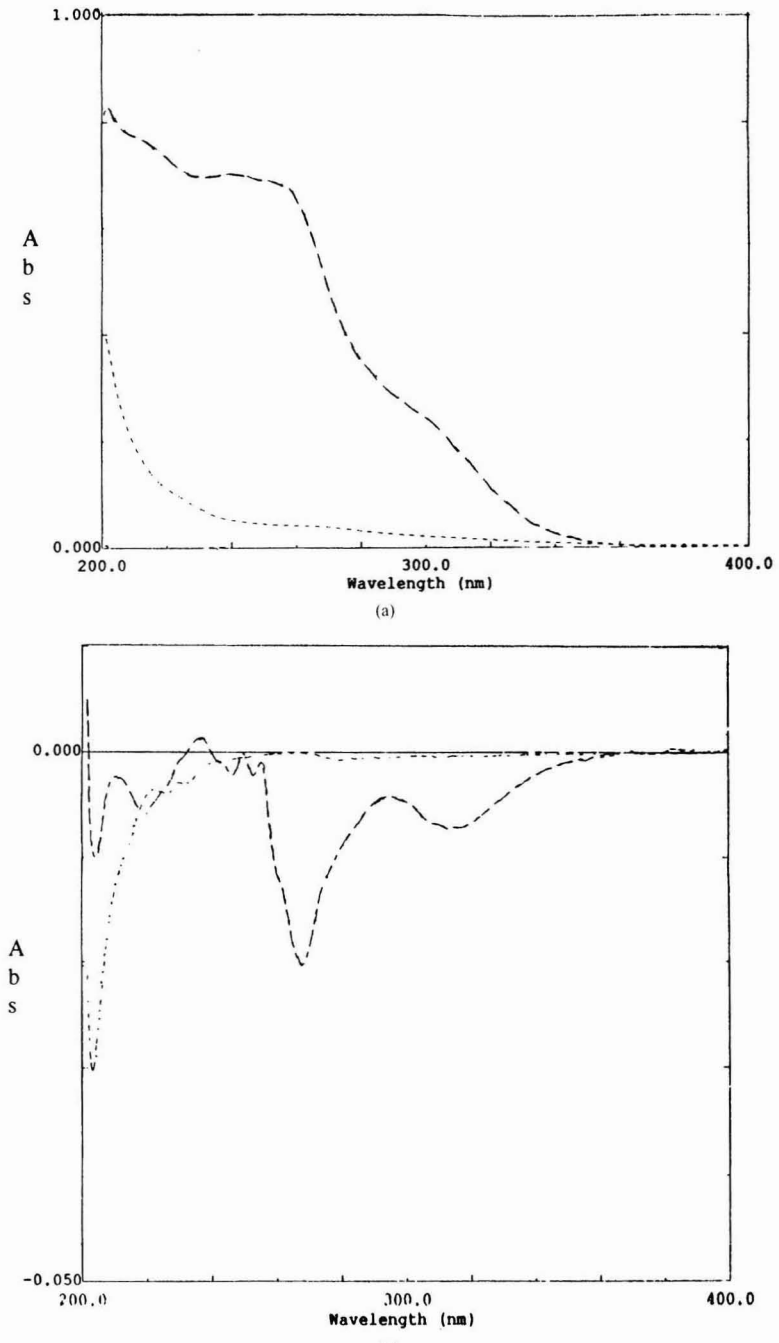
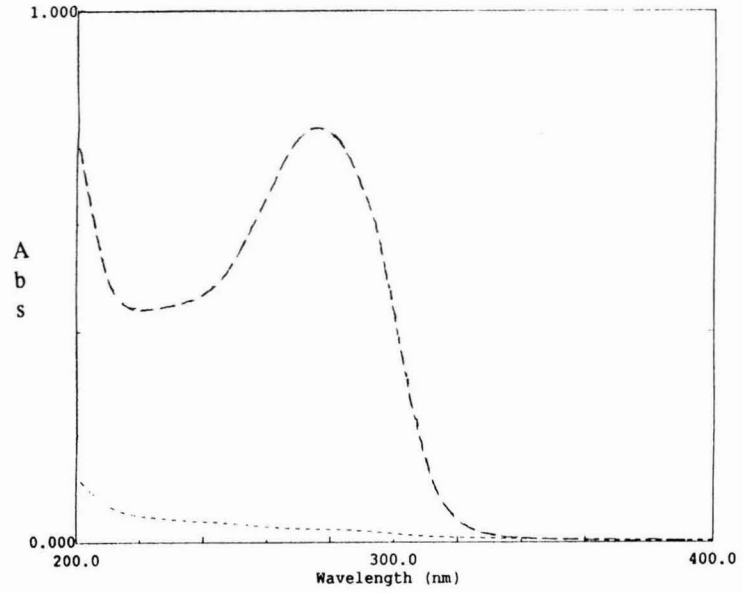
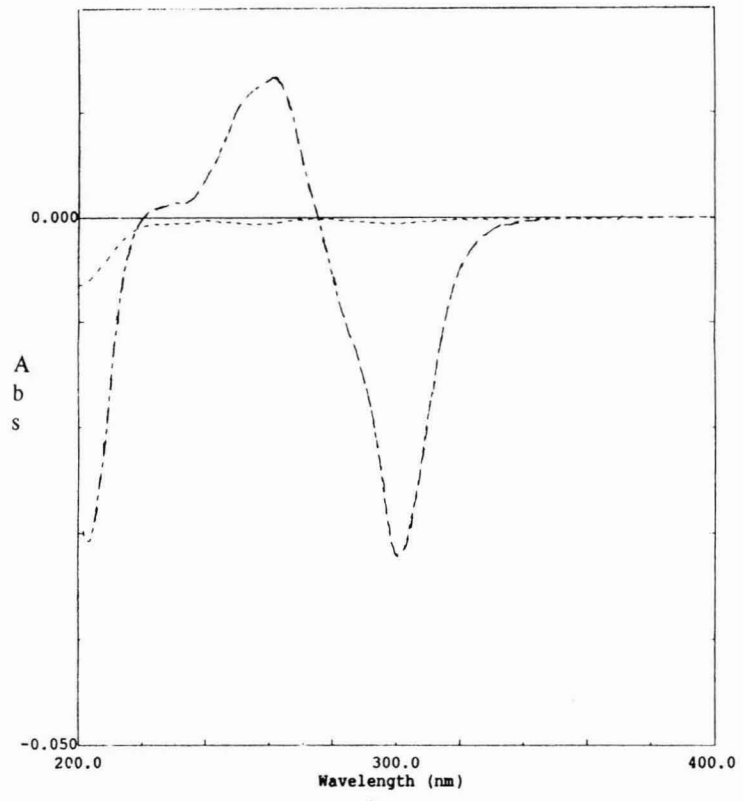


Figure 4. (a) Zero-order absorption spectra of ceftazidime 20 μg mL^{-1} (---) and its degradation product 20 μg mL^{-1} (----). (b) First derivative spectra of ceftazidime 20 μg mL^{-1} (---) and its degradation product 20 μg mL^{-1} (----).



(a)



(b)

Figure 5. (a) Zero-order absorption spectra of cefuroxime sodium 20 μg mL^{-1} (---) and its degradation product 20 μg mL^{-1} (----). (b) First derivative spectra of cefuroxime sodium 20 μg mL^{-1} (---) and its degradation product 20 μg mL^{-1} (----).

The main purpose of this work was to establish stability-indicating methods for determination of some cephalosporins in the presence of their degradation products. Two proposed methods using first derivative spectrophotometry (D_1) and densitometry are suggested.

Zero order absorption spectra (D_0) of I, II, and III and their degradation products showed significant overlapping (Figures 4a, 5a, and 6a). However, the first derivative absorption spectra (D_1) obtained under the same conditions showed zero-crossing points for the degradation products at 268.6, 306, and 228.6 nm for I, II, and III, respectively (Figures 4b, 5b, and 6b). Thus, the D_1 method is suggested for the determination of intact drugs at their respective wavelengths.

Laboratory-prepared mixtures of the drugs and their degradation products were analyzed by the first derivative technique at the 3 wavelengths. The results (Table 1) showed no interference in the presence of degradation products at levels of $\leq 90\%$ for I and II and 80% for III.

In the TLC method, trials to separate the 3 drugs from their degradation products were conducted. Good and complete separation was obtained for I with

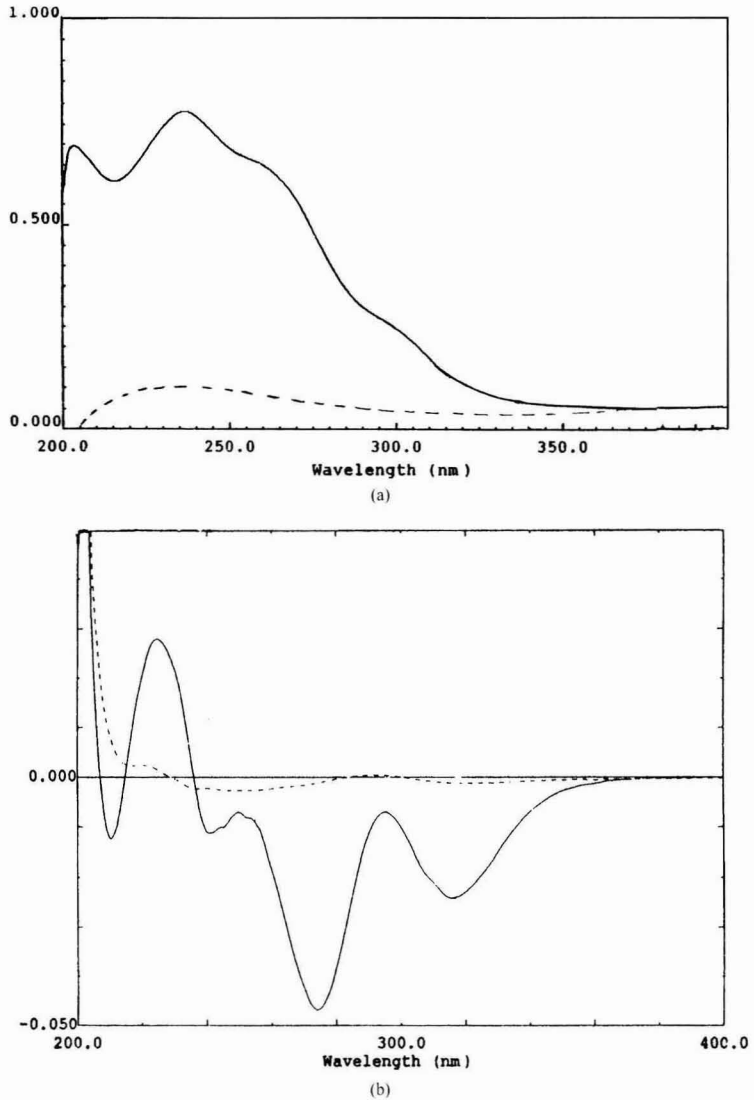


Figure 6. (a) Zero-order absorption spectra of cefotaxime sodium 20 μg mL^{-1} (---) and its degradation product 20 μg mL^{-1} (----). (b) First derivative spectra of cefotaxime sodium 20 μg mL^{-1} (---) and its degradation product 20 μg mL^{-1} (----).

Table 1. Comparison of the proposed methods with the official USP method (14) for the determination of ceftazidime, cefuroxime sodium, and cefotaxime sodium in the presence of their degradation products

		Recovery, %								
		Ceftazidime			Cefuroxime sodium			Cefotaxime sodium		
Sample	Degradation product, %[a]	First derivative	Densitometric method	Official method	First derivative	Densitometric method	Official method	First derivative	Densitometric method	Official method
1	0	99.7	99.5	100.3	100.1	99.2	99.9	99.8	99.7	100.5
2	10	99.1	98.7	99.2	99.6	100.3	98.7	100.7	98.9	99.6
3	20	101.0	100.5	99.7	99.2	100.2	99.1	100.2	99.8	99.2
4	40	100.2	100.7	100.3	98.4	99.4	99.3	99.7	98.2	99.5
5	50	99.1	101.1	98.8	99.7	99.6	99.7	99.5	100.1	98.6
6	70	98.6	99.7	99.5	100.2	100.5	100.4	100.9	101.5	100.2
7	80	100.5	99.9	101.6	100.1	99.8	99.5	101.7	100.6	99.7
8	90	101.3	100.7	99.7	101.4	99.2	100.2	108.5[b]	98.5	99.5
Mean		100.0	100.2	99.8	99.8	99.9	99.6	100.5	99.7	99.5
RSD, %		1.05	0.81	0.91	0.93	0.49	0.60	0.82	1.19	1.30

[a] Calculated with respect to the total weight (drug – degradation mixtures).
[b] Rejected.

Table 2. Comparison of the proposed methods, the official USP method (14), and reported methods for the determination of ceftazidime, cefuroxime sodium, and cefotaxime sodium in pure and dosage forms

Preparation	Found by first derivative method ± RSD, %	Recovery by standard additions ± RSD, %[a]	Found by densitometric method ± RSD, %	Recovery by standard additions ± RSD, %[a]	Found by official and reported methods ± RSD, %
Ceftazidime					
Pure sample	99.7 ± 0.8 t = 1.3 F = 1.78		99.5 ± 0.8 t = 1.78 F = 1.78		100.3 ± 0.6
Fortam for injection, 1 g/vial	99.1 ± 0.4	99.6 ± 0.9	98.4 ± 0.9	100.6 ± 0.5	100.1 ± 0.2
Cefuroxime sodium					
Pure sample	100.1 ± 0.7 t = 0.59 F = 5.4		99.2 ± 0.7 t = 2.05 F = 5.4		99.9 ± 0.3
Zinnat tablets, 0.25 g/capsule	99.4 ± 0.4	99.7 ± 0.5	99.2 ± 0.9	99.6 ± 0.3	100.5 ± 0.7
Zinnat suspension 125 mg/5 mL	99.5 ± 0.4	100.5 ± 1.2	100.5 ± 1.2	99.5 ± 0.9	99.2 ± 1.1[b]
Cefotaxime sodium					
Pure sample	99.8 ± 0.8 t = 1.75 F = 4.0		99.7 ± 0.8 t = 1.9 F = 2.56		100.5 ± 0.5
Claforan for injection,					
0.25 g/vial	98.3 ± 0.6	100.0 ± 0.3	99.4 ± 0.4	99.2 ± 0.3	99.9 ± 0.2[c]
0.5 g/vial	99.1 ± 0.3	99.7 ± 0.1	100.1 ± 0.2	99.5 ± 0.4	99.4 ± 0.3[c]
1 g/vial	99.5 ± 0.6	99.9 ± 0.5	99.7 ± 0.3	99.1 ± 0.6	99.6 ± 0.4[c]

[a] Average of 5 analyses.
[b] Glaxo, personal communication.
[c] Hoechst, personal communication.

methanol–1N acetic acid (25 + 75, v/v) as the mobile phase and for II and III with methanol-concentrated ammonium hydroxide (100 + 1.5, v/v) as the mobile phase. The R_f values for I, II, and III were 0.45, 0.93, and 0.85, respectively, whereas those of the degradation products were 0.9, 0.75, and 0.7, respectively. The chromatograms were scanned quantitatively at 258, 272, and 262 nm for I, II, and III, respectively.

When this technique for determining the 3 drugs in the presence of their degradation products was applied to laboratory-prepared mixtures, no interferences were found in the presence of degradation products at levels of $\leq 90\%$. The results are shown in Table 1.

Calibration

The linearity of the response obtained by derivative spectrophotometry was evaluated by analyzing standard solutions in concentration ranges of 5–50, 5–35, and 5–40 μg/mL for I, II, and III, respectively. The typical standard curves were obtained.

The data fit the regression straight lines represented by the following equations: $D_1 = 0.0004 + 0.0011C$, $r = 0.9998$ for I; $D_1 = 0.0001 + 0.00145C$, $r = 0.9995$ for II; and $D_1 = 0.0012 - 0.0002C$, $r = 0.9998$ for III, where D_1 is the first derivative value, C is the concentration in μg/mL and r is the correlation coefficient.

The concentration range for the densitometric determination was 4–16 μg for I and 2–12 μg for II and III. The data fit the regression straight lines represented by the following equations $A = 1.038C - 0.359$, $r = 0.9992$ for I; $A = 0.615 + 0.955C$, $r = 0.9981$ for II; and $A = 1.05C - 0.24$, $r = 0.9962$ for III, where A is the area under the peak, C is the concentration in mg/mL, and r is the correlation coefficient. The calibration graphs were linear with these correlation coefficients.

Precision

Precision was evaluated by performing 5 analyses of each sample. The relative standard deviations (RSDs) were 0.8, 0.7, and 0.8% with derivative spectrophotometry and 0.8, 0.7, and 0.8% with the densitometric method for I, II, and III, respectively.

The proposed methods were successfully applied to the determination of the 3 drugs in pharmaceutical dosage forms. The results obtained were compared statistically with those obtained by applying the USP method (14) and reported methods (Glaxo, personal communications; Hoechst, personal communication) (Table 2).

Method Validation

The validity of the proposed methods was assessed by applying the technique of standard additions. The results are presented in Table 2.

Conclusions

Table 2 shows that the calculated t and F values are less than the corresponding theoretical values, indicating that there is no significant difference between the 2 methods with respect to both precision and accuracy.

These results substantiate the usefulness of the 2 suggested methods for the determination of ceftazidime, cefuroxime sodium, and cefotaxime sodium in pure materials or in pharmaceutical dosage forms. They have the advantage of being applicable in the presence of degradation products.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Optical Immunobiosensor Assay for Determining Enrofloxacin and Ciprofloxacin in Bovine Milk

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A rapid, sensitive optical immunobiosensor assay was developed and used to determine enrofloxacin and its main metabolite, ciprofloxacin, in milk from healthy cows and cows with clinical signs of mastitis after intramuscular administration of enrofloxacin. Liquid chromatography (LC) was used to confirm results of the biosensor assay. Despite incomplete cross-reactivity between polyclonal enrofloxacin antibodies and ciprofloxacin, the biosensor assay could be used for semiquantitative analysis of the sum of the 2 substances. LC analysis showed that ciprofloxacin persisted at levels exceeding the expected future maximum residue limit in milk for several days after the end of the withdrawal period.

Enrofloxacin is a synthetic antimicrobial agent of the fluoroquinolone family, a group of gyrase inhibitors recognized by their large volume of distribution and broad bactericidal and mycoplasmacidal activity at very low concentrations (1). Whereas some fluoroquinolones, such as ciprofloxacin, are restricted for human use, enrofloxacin has been specially designed for use in veterinary medicine.

In Sweden, enrofloxacin is used in mastitis therapy against infections caused by gram-negative bacteria, such as *Escherichia coli*. In the cow, a large proportion of enrofloxacin is deethylated to ciprofloxacin, which, unlike enrofloxacin, has a long elimination time in milk (2). In Sweden at present, the withholding time for milk after treatment with enrofloxacin is 3 days. There is still no maximum residue limit (MRL) for enrofloxacin in milk. However, considering the acceptable daily intake and MRLs for muscle, liver, and kidney (30 μg/kg), an MRL for the sum of enrofloxacin and ciprofloxacin in milk is expected to be fixed at concentrations lower than 10 μg/kg (3).

In general, microbiological inhibitor assays for routine control of inhibitory substances in milk fail to detect fluoroquinolone residues at sufficiently low levels (4). In this study, a rapid, sensitive optical biosensor assay was developed to study the elimination of enrofloxacin and ciprofloxacin in milk from healthy cows and cows with clinical mastitis after treatment with enrofloxacin. Liquid chromatography (LC) was used to confirm biosensor assay results.

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Experimental

Biospecific Interaction Analysis

(a) *Instrumentation.*—Biospecific interaction analysis (BIA) was conducted on a surface plasmon resonance biosensor, the BIACORE™ system (Biacore AB, Uppsala, Sweden). In this instrument, binding of analyte to ligand takes place on the surface of an exchangeable sensor chip (5). The binding event causes a change in the refractive index on the surface and thereby also a shift in the resonance angle, which can be used for biosensing purposes (6).

(b) *Reagents.*—Polyclonal antibodies were produced by Philipp Hammer (Federal Dairy Research Institute, Kiel, Germany) as previously described (3). Antibodies were prepared from serum by ammonium sulfate precipitation before dialysis against 10 mM phosphate buffer, pH 7.5, with 0.15M NaCl. Sensor chip CM5, HBS buffer pH 7.4 [consisting of 10 mM 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid (Hepes), 150 mM NaCl, 3.4 mM EDTA, and 0.005% (v/v) Surfactant P 20], amine coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), ethylenediamine, and a 1M solution of ethanolamine, pH 8.5 were obtained from Biacore AB. *N,N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Enrofloxacin and ciprofloxacin were kindly supplied by Bayer AG (Leverkusen, Germany). All other reagents were of analytical grades.

(c) *Sensor surface.*—The ligand was immobilized in 2 steps. First, enrofloxacin was esterified with NHS in the presence of EDC. Enrofloxacin (14 mg), NHS (4 mg), and EDC (2 mg) were dissolved in 1.5 mL dry DMF and 3.5 mL DMSO and stired overnight at 4°C. Then, the carboxymethylated dextran surface was activated by contact with 30 μL NHS–EDC (1 + 1) for 10 min, followed by amine coupling with ethylenedi-

amine (30 μL) for 12 min. The esterified enrofloxacin was diluted 1:2 with 25 mM phosphate buffer (pH 7.5), and 30 μL of the solution was applied to the surface (contact time, 30 min). Finally, the surface was blocked with 30 μL 20 mM NaOH (contact time, 5 min). HBS buffer was used to wash the surface between every step of the immobilization procedure. All steps were performed on the bench.

(d) *Preparation of standards.*—Stock solutions of enrofloxacin (1 mg/mL) for use in standards were prepared in 0.18M NaOH. Further dilutions of the stock solutions were made in 30 mM NaOH, and the final 1:100 dilutions were made in milk to reach concentrations of 1–10 μg/kg. A standard curve was constructed by least-square linear regression of enrofloxacin concentration vs response.

(e) *Assay.*—A competitive enzyme immunoassay was used, analogous to a previously described BIA assay for detection of sulphamethazine (7, 8). Polyclonal antibodies (10 nM) against enrofloxacin were added to defatted milk sample, and the mixture (30 μL) was injected across the immobilized sensor surface at a flow rate of 5 μL/min. The instrument measures the amount of free antibodies binding to the surface, and the response is inversely proportional to the concentration of the analyte in the sample. For quantitation, the sample response was compared with the standard curve. The next sample was injected after surface was regenerated with 15 μL 0.18M NaOH at 5 μL/min.

Liquid Chromatography

(a) *Instrumentation.*—Analysis was performed on a Merck Hitachi (Darmstadt, Germany) high-pressure LC system consisting of La Chrome L-7100 delivery system and LaChrome L-7480 fluorescence detector. Wavelength settings were 278 nm for excitation and 440 nm for emission. Analytical column (150 × 4.6 mm) and guard column (5.0 × 3.0 mm) were packed with 5 μm particles of PLRP-S polymer adsorbent (Polymer Laboratories, Amherst, MA). Integration was performed with the software program JCL 6000 (Jones Chromatography, Mid-Glamorgan, UK).

(b) *Reagents.*—All chemicals were analytical or HPLC grade. Heptane sulfonic acid was obtained from Sigma Chemicals Co. (St. Louis, MO) and phosphoric acid was from Riedel-de Haen (Seelze, Germany). Acetonitrile and methanol were purchased from Merck.

(c) *Method.*—Liquid chromatography was performed according to a method described by Hormazábal and Yndestad (9). The mobile phase was a mixture of three solutions, A, B, and C (68 + 24 + 8). Solution A consisted of 0.02M heptane sulfonate in 0.002M phosphoric acid; solution B was acetonitrile; and solution C was methanol. The flow rate was 1 mL/min, and 30 μL sample was injected onto the column. Retention times were 6.2 and 8 min for enrofloxacin and ciprofloxacin, respectively.

(d) *Preparation of standards.*—Stock solutions (1 mg/mL) for use as enrofloxacin and ciprofloxacin standards were prepared in 0.18M NaOH. Further dilutions were made in 30 mM NaOH, and final 1:100 dilutions were made in mobile phase.

(e) *Sample preparation.*—Samples were prepared by molecular mass cutoff filtration according to the method described by Tyczkowska et al. (10). Milk samples were defatted and mixed with an equal volume of acetonitrile–0.1M NaOH (1 + 1). Portions (2 mL) of the mixture were centrifuged in a 5000 Da molecular mass cutoff filter (Ultrafree-CL filters, Millipore, Bedford, MA) for 50 min at 4000 × *g*. Ultrafiltrates (30 μL) were injected onto the chromatographic system, and quantitation was done by comparison with standards containing 5 and 50 μg enrofloxacin and ciprofloxacin/kg.

Milk Samples

(a) *Milk from healthy cows.*—Enrofloxacin (Baytril®, Bayer AG) was given to three cows (A, B, and C) with no signs of clinical or subclinical mastitis at the Department of Cattle and Sheep Diseases (Swedish University of Agricultural Sciences, Uppsala). The drug was administered intramuscularly at a dosage of 2.5 mg/kg body weight for 3 consecutive days. Milk samples from each udder quarter were collected twice daily from the first day of treatment and for 8 successive days. Samples were stored at −20°C prior to analysis.

(b) *Milk from cows with clinical mastitis.*—Enrofloxacin (Baytril) was administered to three cows (D, E, and F) with clinical mastitis intramuscularly at a dosage of 2.5 mg/kg body weight for 3 consecutive days. Cow F was treated for 2 days only, and then the therapy was modified in accordance with results of bacteriological examination. The cows were from different dairy herds in Uppsala and were being treated by veterinarians at the Ambulatory Clinic, Department of Obstetrics and Gynaecology (Swedish University of Agricultural Sciences, Uppsala). Milk samples were collected by the farmer responsible during ordinary morning and evening milkings from the first day after the last treatment and for 7 consecutive days for cows D and E and 5 consecutive days for cow F. Samples from cow D were produced by pooling milk from 3 uninfected quarters. For cows E and F, samples were produced by pooling milk from all 4 quarters. Samples were stored at −20°C prior to analysis.

Results and Discussion

Milk samples from all 6 cows were analyzed by BIA, and results were confirmed by LC, exception results of samples from cow E. Because the LC system was not

available at the time of analysis of milk from cow E, these milk samples were analyzed only by BIA.

The limit of detection of the immunobiosensor assay was 1.5 μg/kg for both enrofloxacin and ciprofloxacin, and the coefficient of variation (n = 7) was 2.5%. The LC method produced a standard curve that was linear from 5 to 100 μg/kg for both enrofloxacin and ciprofloxacin. Detection limits were about 2 and 3 μg/kg for enrofloxacin and ciprofloxacin, respectively. At 32 μg/kg, coefficients of variation (n = 10) were 8.3 and 7.5% and recoveries were 90 and 86% for enrofloxacin and ciprofloxacin, respectively.

Concentrations of enrofloxacin and ciprofloxacin in milk from healthy cows treated with enrofloxacin were analyzed per quarter (data not shown). Figure 1 shows average concentrations of enrofloxacin and ciprofloxacin in milk from cows A, B, and C as determined by BIA and LC.

Enrofloxacin concentration dropped to 6 μg/kg during the first day after the last treatment. Ciprofloxacin concentration, however, was 21 μg/kg on the third day after the last treatment, that is, at the end of the withholding time. On the seventh day after the last treatment, ciprofloxacin levels were still high (26 μg/kg). Analysis by BIA gave an estimate of the sum of enrofloxacin and ciprofloxacin in the milk. Because the polyclonal enrofloxacin antibodies cross-react <100% with ciprofloxacin (11), concentrations obtained by BIA were lower than those obtained by LC.

Figure 2 shows average concentrations of enrofloxacin and ciprofloxacin, as determined by BIA and LC, in composite milk samples from the 3 cows with clinical mastitis. Enrofloxacin concentration was <10 μg/kg on the second day after the last treatment, whereas ciprofloxacin concentration was as high as 44 μg/kg at 3 days after the last treatment, that is, at the end of the withholding period. Even at 5 days after the last treatment, ciprofloxacin persisted at relatively high levels (10 μg/kg).

The present study shows that enrofloxacin rapidly metabolizes to ciprofloxacin, which takes a long time to be eliminated from milk in both healthy cows and cows with clinical mastitis. This finding agrees with previous studies. Kaartinen et al. (2) concluded that ciprofloxacin was trapped in milk after treatment of cows with enrofloxacin, and Saraste et al. (4) found that the sum of enrofloxacin and ciprofloxacin remained at high levels beyond the 4 days withholding time in Finland.

The low minimum inhibitory concentrations of fluoroquinolones against many gram-negative bacteria, for which the choice of antimicrobials otherwise is limited, is one reason for using these very potent drugs in mastitis therapy. This study and others indicate the necessity to reevaluate existing withholding times for milk to comply with regulatory residue limits. Considering the risk for development of resistant bacteria and the importance of ciprofloxacin in human therapy, use of enrofloxacin in lactating cows should be restricted.

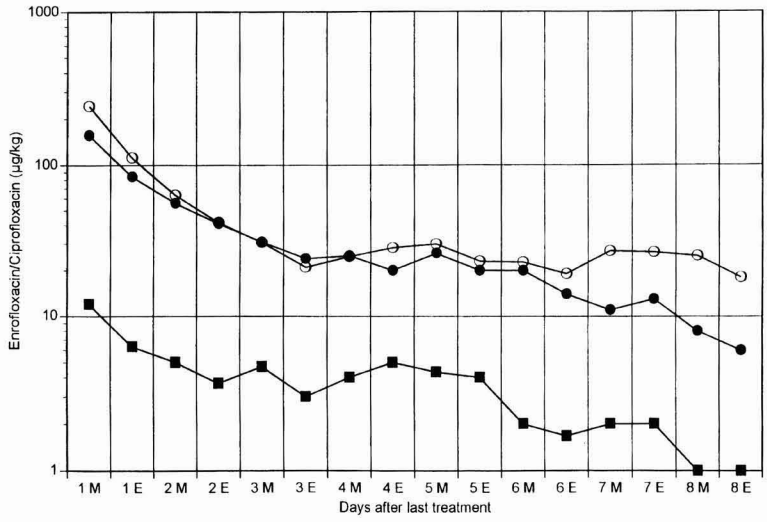

Figure 1. Average concentrations of enrofloxacin (EF) and ciprofloxacin (CF), as determined by biospecific interaction analysis (BIA) and liquid chromatography, in milk from 3 cows with no signs of clinical mastitis. Cows were treated for 3 days with enrofloxacin, and the figure shows average concentrations from the first day after the last treatment. M = morning milking; E = evening milking; ● = BIA; ○ = liquid chromatography (CF); ■ = liquid chromatography (EF).

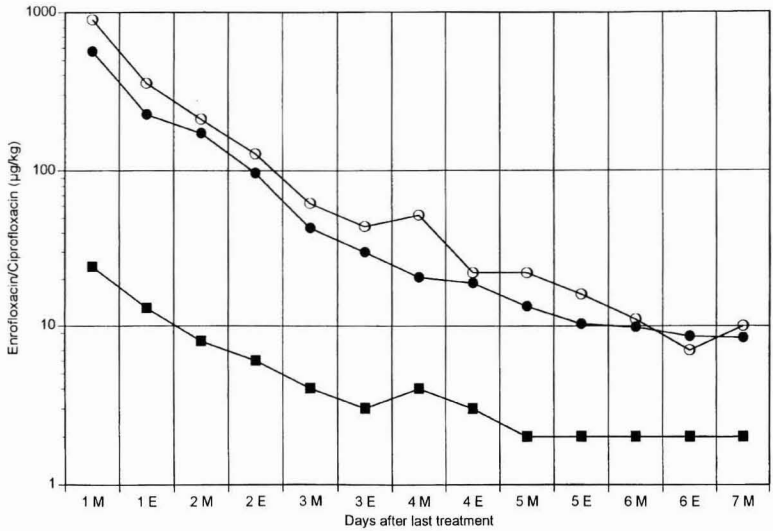

Figure 2. Average concentrations of enrofloxacin (EF) and ciprofloxacin (CF), as determined by biospecific interaction analysis (BIA) and liquid chromatography, in milk from 3 cows with clinical mastitis. The cows were treated for 3 days with enrofloxacin, and the figure shows average concentrations from the first day after the last treatment. M = morning milking; E = evening milking; ● = BIA, ○ = liquid chromatography (CF); ■ = liquid chromatography (EF).

The low sensitivity of microbial inhibitor assays to fluoroquinolones is another problem. Detection limits of commercial tests used in routine control for inhibitory substances in milk usually are at the mg/kg level. These levels must be considered unsatisfactory given the toxicity of these substances. In the study of Saraste et al. (4), the microbial inhibitor tests T101 (Valio, Helsinki, Finland) and Delvotest SP (Gist-Brocades, Delft, The Netherlands) showed positive results at total concentrations of enrofloxacin and ciprofloxacin between 401 and 1204 µg/kg.

The results of this study indicate the need for sensitive and specific screening assays to complement conventional inhibitor assays for assessing both the technological and the toxicological qualities of raw milk. The biosensor assay used in this study is very sensitive, rapid, and fully automated and may be useful as a screening method for both assessments.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Comparison of Monolayer and Bilayer Plates Used in Antibiotic Assay

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Standard curves of 5 antibiotics were determined in an antibiotic assay using bilayer and monolayer agar plates and AOAC-specified test organisms and agar media. *Micrococcus luteus* ATCC 9341a and antibiotic medium No. 2 were used to prepare the penicillin G standard curve. The same organism and antibiotic medium No. 11 were used to prepare the erythromycin standard curve. Standard curves for streptomycin, tetracycline, and gentamicin were prepared, respectively, with antibiotic medium No. 5 and *Bacillus subtilis* ATCC 6633, antibiotic medium No. 8 and *B. cereus* ATCC 11778, and antibiotic medium No. 11 and *Staphylococcus epidermidis* ATCC 12228. Assays of inhibition by meat fortified with penicillin, streptomycin, gentamicin, tetracycline, erythromycin also were performed on monolayer and bilayer plates. Differences in standard curves and inhibitory responses obtained with monolayer and bilayer plates were <10%. Thus, monolayer plates are acceptable for use in analyses of meat and poultry for antibiotics residues, with savings in laboratory resources and time.

The cylinder cup agar plate diffusion assay is used routinely for determining antibiotic potency (1). A dose line derived from the assay, known as the standard curve, shows the relationship between antibiotic concentration and zone of inhibition against a test microorganism. Among other factors, specificity and concentration of the test organism and volume of the assay agar affect the analysis.

The assay also has been used for determining antibiotic concentrations in animal feed (2–10). Traditionally, double or bilayer plates have been used. These consist of a base agar layer covered with another agar layer containing a specific number of organisms (1).

Monolayer plates used in antibiotic assay consist of a single agar layer containing a specific number of organisms. Use of monolayer plates requires less labor, materials, and time, and the analytical performance (sensitivity and accuracy) is equal to or better than with use of bilayer plates (11). Further, monolayer plates provide greater ease of use, better flexibility in obtaining optimal sensitivity, and improved standardization of the methodology (11). Use of monolayer plates would be acceptable provided the inhibitory zone of an antibiotic standard curve reference concentration on monolayer plates is within ±10% of the zone on bilayer plates (1).

The agar diffusion technique is used by the U.S. Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) for routine analysis of thousands of food animal tissue samples for antibiotic residues. The assay is also used to develop methods for detecting and identifying new antibiotics used in food animals. Use of bilayer plates is labor intensive and time consuming.

The present USDA/FSIS antibiotic assay procedure requires that a meat tissue be diluted 5-fold in 3 phosphate buffer solutions of pH 4.5, 6.0, and 8.0 (12). Therefore, assay plates should be sensitive enough to detect violative levels of antibiotic residues in the diluted tissue. The antibiotics and their violative concentrations are determined by the U.S. Food and Drug Administration and listed in the Federal Code of Regulations.

Use of monolayer plates reduces the time required to prepare plates and saves a significant amount of resources. However, such change should not interfere with the sensitivity of the assay for detecting violative levels of antibiotic residues in meat.

The study compares the performance of monolayer plates with bilayer plates on the basis of standard curve values of 5 antibiotics and inhibitory responses of antibiotic-fortified meat.

Experimental

Organisms

Test organisms were *Micrococcus luteus* (MLA) ATCC 9341a, *Bacillus subtilis* (BS) ATCC 6633, *B.*

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cereus (BC) ATCC 11778, and *Staphylococcus epidermidis* (SE) ATCC 12228. Spores of BS, catalogue No. 0453-60, and BC, catalogue No. 0959-52, were obtained from Difco, Detroit, MI. Cultures of SE and MLA were cryopreserved in methylcellulose (13).

Media and General Preparation of Plates

Antibiotic medium No. 2 (catalogue No. 10912), No. 5 (catalogue No. 10953), and No. 8 (catalogue No. 10965) were prepared according to manufacturer's (Beckton and Dickinson, Cockeysville, MD) directions.

Bilayer plates were prepared by pipetting 10 mL agar into each plate. After the agar had solidified, the entire surface was covered with 4.0 mL of the same agar seeded with a known number of organisms.

Monolayer plates were prepared by pouring into each plate 8.0 mL test agar seeded with a known number of test organism.

Antibiotic Standards

Penicillin G (catalogue No. P-7794), streptomycin sulfate (catalogue No. S-6501), erythromycin (catalogue No. E-6376), tetracycline (catalogue No. T-3383), and gentamicin (catalogue No. G-3632) were obtained from Sigma Chemical Co., St. Louis, MO.

Preparation of Specific Plates

(a) *BC plates.*—A 0.15 mL portion of BC spore suspension (2.5×10^7 spores/mL) was added to 100 mL antibiotic medium No. 8 kept in a 48°C water bath and mixed gently. After the 45 min incubation required to germinate BC spores before plates are prepared, 8.0 mL agar was pipetted into each plate (100 × 15 mm) for a monolayer plate. For a bilayer plate, 4.0 mL was pipetted over a 10.0 mL base layer of antibiotic medium No. 8. Plates were refrigerated and discarded after 5 days.

(b) *BS plates.*—Monolayer and bilayer plates were prepared as described above, except 100 mL antibiotic medium No. 5 was seeded with 0.5 mL BS spore suspension (2.5×10^7 spores/mL) and incubated for 75 min in a 48°C water bath to allow BS spores to germinate before plates are prepared.

(c) *MLA-2 plates.*—Plates were prepared as described above, except 100 mL antibiotic medium No. 2 was seeded with 0.15 mL MLA (1×10^8/mL) with no preincubation in the water bath.

(d) *MLA-11 plates.*—Plates were prepared as described in (c) above, except agar was replaced with antibiotic medium No. 11.

(e) *SE plates.*—Plates were prepared as described in (d) above, except antibiotic medium No. 11 was seeded with 0.15 mL SE (1×10^8/mL).

Preparation of Antibiotic Standards

AOAC-recommended buffers were used for preparing standard solutions of each of the test antibiotics (14).

A stock solution (1000 μg/mL) of penicillin G was made in pH 6.0 phosphate buffer. Working standards (0.0125, 0.025, 0.05, 0.1, and 0.2 μg/mL), also in pH 6.0 phosphate buffer, were prepared from stock solution.

Similarly, working standards of gentamicin sulfate (0.08, 0.16, 0.32, 0.64, and 1.28 μg/mL) and streptomycin sulfate (0.125, 0.25, 0.5, 1.0, and 2.0 μg/mL) were prepared from stock solution in pH 8.0 phosphate buffer. Working standards of erythromycin sulfate (0.05, 0.1, 0.2, 0.4, and 0.8 μg/mL) prepared in pH 8.0 buffer from a stock solution (1000 μg/mL) were made by dissolving the salt in 2 mL methanol and then diluting with pH 8.0 phosphate buffer to a final concentration of 1000 μg/mL. Tetracycline (0.08, 0.16, 0.32, 0.64, and 1.28 μg/mL) working standards were prepared from stock solution (1000 μg/mL), which was prepared by dissolving the salt in 2 mL 1N HCl and diluting with pH 4.5 phosphate buffer to a final concentration of 1000 μg/mL.

The first and the third working solutions of all antibiotics were used as the minimum inhibitory and the reference concentrations, respectively.

Determination of Standard Curves

Stainless steel bioassay spiders with 6 wells (Arthur E. Farmer, Trenton, NJ) were placed on each of 12 monolayer and bilayer plates containing antibiotic agar No. 2. Two hundred microliters of one concentration of working penicillin G solution was pipetted into 3 alternate wells of spiders. In the 3 other wells, 200 μL reference (i.e., the third concentration of the standard solution) was pipetted. Similarly, other plates were inoculated with other concentrations of standards and reference solutions. After incubation at 37°C for 18 h, spiders were removed and the zone of inhibition on each plate was recorded. Standard graphs from monolayer and bilayer plate data were prepared with an FSIS-developed computer program based on AOAC standard-curve methodology (15).

Similarly, standard graphs of monolayer and bilayer plate data for streptomycin, tetracycline, gentamicin, and erythromycin were generated by computer.

Inhibition of Test Microorganisms by Animal Tissue Fortified with Antibiotic

As described in the FSIS laboratory guidebook (12), muscle tissue fortified with a known quantity of penicillin was extracted in pH 6.0 phosphate buffer. By using steel spiders, a set of monolayer and bilayer plates seeded with MLA were inoculated with the meat extract. After plates were incubated at 37°C for 18 h, zones of inhibition produced on both plate types were recorded. Similarly, zones of inhibition on both plate

types caused by extracts of meat fortified with other antibiotics were recorded.

Results

Computer-generated values for y intercepts, slopes, and mean references for 5 antibiotic standard curves for monolayer and bilayer plate data are presented in Table 1.

Bilayer plate data gave higher values of the logarithm of the y intercept for all antibiotics except streptomycin and erythromycin than values from monolayer plate data. Slopes for monolayer and bilayer plate data for all antibiotics differed by ± 0.1, except for penicillin and tetracycline. The difference in the mean reference values for monolayer and bilayer plate data for all antibiotics was highest for penicillin G (9.7%) and lowest for streptomycin (2.3%).

The difference in standard curve values indicates that the responses of monolayer plates, as judged from the zones of inhibition by concentrations of test antibiotics, were different from those of bilayer plates. However, on the basis of mean values of reference zones, the differences in the responses monolayer and bilayer plates were within the acceptable 10% limit.

By using the same program (15), standard curves of monolayer and bilayer plate data for all antibiotics were computer generated, an example of which is presented in Figure 1. Standard curve data for all other antibiotics were similar. Zones of inhibition produced by extracts of antibiotic-fortified muscle tissues on monolayer and bilayer plates are presented in Table 2. The average difference in the zones of inhibition on monolayer and bilayer plates for the antibiotics ranged from 0.2 to 0.5 mm. Except for streptomycin, zone sizes for all antibiotics were higher with monolayer plates than with bilayer plates.

Statistical Analysis of Data

Mean reference values (Table 1) were analyzed further by the paired t-test to determine if differences were significant (16). With 8 degrees of freedom, the critical value for a 2-tailed test was ± 2.306 at the 0.05 significance level. The t values were calculated on the basis of mean difference between the paired values and their standard deviation (Table 3). Values for streptomycin (1.45) and erythromycin (2.01) indicate that the differences between monolayer and bilayer values for the 2 antibiotics were not significant at the 5% level. However, t values of -7.70 for penicillin G, 5.15 for tetracycline, and -2.96 for gentamicin indicate that the differences between monolayer and bilayer values were significant at the 5% level. Values for penicillin and gentamicin indicate that monolayer plates are more sensitive to these antibiotics whereas bilayer plates are more sensitive to tetracycline. However, the differences in mean reference values (Table 1) indicates that the differences noted (Table 3) between monolayer and bilayer plate values do not differ by more than 10%.

Zones of inhibition produced by muscle tissue extracts fortified with various antibiotics on monolayer and bilayer plates were analyzed statistically. Differences in inhibition were not significant at the 5% level (Table 2).

Discussion

The agar diffusion plate assay for antibiotics is usually performed with plates containing 2 layers of agar. Among many factors, the amount of agar affects the sensitivity of the assay. Thus, a specific amount of agar is recommended to achieve the desired level of assay sensitivity (17).

The main purpose of the study was to determine if bilayer plates could be replaced with monolayer plates. If so, then monolayer plates, which require less time and material and are easy to prepare, could be used by USDA/FSIS laboratories without compromising the efficacy of the assay used to monitor antibiotic residues in meat and poultry. As the agency checks thousands of meat samples for antibiotic residues annually, use of monolayer plates would save enormous amount of time and resources.

The AOAC standard-curve assay procedure was chosen to avoid discrepancy arising from methodology for comparing assay responses with monolayer and bilayer plates. The study shows that the response of monolayer plates to streptomycin and erythromycin does not differ significantly from that of the bilayer plates at the 5% level. Although the response of mono-

Table 1. Logarithm of y intercept, slope, and mean reference values of standard curves for various antibiotics obtained with bilayer (BL) and monolayer (ML) plates

	Log of y intercept		Slope		Mean reference		
Antibiotic	BL	ML	BL	ML	BL	ML	Difference, %
Streptomycin	−2.2430	−1.9518	0.1048	0.0948	17.6	17.2	2.3
Penicillin G	−2.9804	−3.6039	0.0795	0.1010	17.5	19.2	9.7
Tetracycline	−1.9920	−2.0215	0.0788	0.0878	19.5	17.9	8.2
Gentamicin	−2.2223	−2.2275	0.1053	0.1001	17.0	18.0	5.9
Erythromycin	−2.6778	−2.6286	0.1088	0.1123	18.5	17.7	4.3

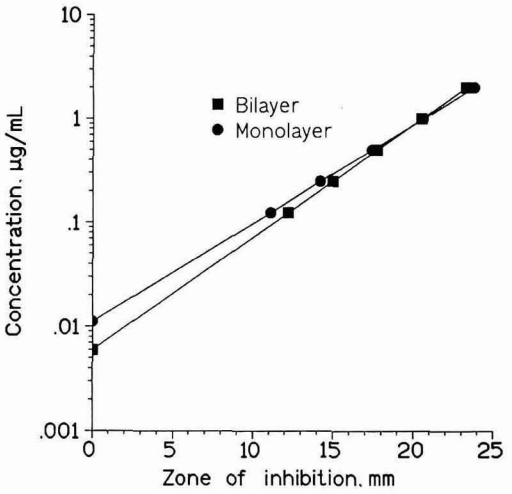

Figure 1. Standard curves for streptomycin.

layer plates to penicillin, tetracycline, and gentamicin vary from that of bilayer plates by more than 5%, the differences are within 10%. This change in response was expected because the volume of agar directly affects the diffusion of antibiotic.

This study and earlier pilot studies show that the performance of monolayer plates, like bilayer plates, varies with agar smoothness, uniformity, and depth. Performance does not change for 5 days if plates are sleeved and stored at 4°C. However, for optimum performance, freshly prepared plates should be tested with reference concentrations of antibiotics before use in analysis. Although, 8.0 mL agar used in monolayer plates provides adequate support for spiders, careless placing can tear the agar surface.

The increased sensitivity of monolayer plates for some antibiotics was another positive outcome. This higher sensitivity, resulting from the quantity of agar used in monolayer plates, allows federal laboratories to detect antibiotics, usually in microgram quantities, as violative levels set by the Federal Code of Regulations. Additionally, it has been reported that assay plates easily can be adjusted by manipulating the concentration of organisms in the agar (11, 17). Thus, USDA/FSIS laboratories have been able to adjust assay sensitivity by regulating the sensitivity of plates. The simple adjustment has helped USDA/FSIS develop methods for detecting violative levels of practically all antibiotics used in food animals.

Table 2. Zone of inhibition by antibiotics in muscle tissue on monolayer and bilayer plates

	Mean zone of inhibition, mm (n = 9)	
Antibiotic	Monolayer plate	Bilayer plate
Penicillin	15.5	15.0
Streptomycin	10.0	10.2
Erythromycin	16.5	16.0
Tetracycline	11.8	11.4
Gentamicin	12.5	12.0

Table 3. Statistical analysis of monolayer and bilayer reference values of antibiotic curves

	Differences		
Antibiotic	Mean	Standard deviation	t value
Streptomycin	0.38	0.806	1.45
Penicillin G	−1.71	0.666	−7.70
Tetracycline	1.58	0.918	5.15
Gentamicin	1.00	1.012	−2.96
Erythromycin	0.83	1.240	2.01

Since 1992, monolayer plates have been used routinely in all USDA/FSIS laboratories for analysis of antibiotic residues. Adaptation of monolayer plates has saved resources and has improved the analytical capabilities of FSIS in antibiotic residue detection.

Conclusion

The study suggests that antibiotic standard curves derived from use of monolayer and bilayer plates are not significantly different. The study also indicates that inhibition of test organism in agar cup diffusion technique by the antibiotic residue in meat tissue on monolayer plates is not significantly different from those observed on bilayer plates. The data indicate that monolayer plates are acceptable for use in analyses of meat and poultry tissues for antibiotic residues.

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FOOD BIOLOGICAL CONTAMINANTS

Direct 24-Hour Presumptive Enumeration of *Escherichia coli* O157:H7 in Foods Using Hydrophobic Grid Membrane Filter Followed by Serological Confirmation: Collaborative Study

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Fifteen laboratories took part in a collaborative study to validate a method for enumerating *Escherichia coli* O157:H7. The method is based on use of a hydrophobic grid membrane filter and consists of 24 h presumptive enumeration on SD-39 Agar and serological confirmation to yield a confirmed *E. coli* O157:H7 count. Six food products were analyzed: pasteurized apple cider, pasteurized 2% milk, cottage cheese, cooked ground pork, raw ground beef, and frozen whole egg. The test method produced significantly higher confirmed count results than did the reference method for milk, pork, and beef. Test method results were numerically higher than but statistically equivalent to reference method results for cheese, cider, and egg. The test method produced lower repeatability and reproducibility values than did the reference method for most food/inoculation level combinations and values very similar to those of the reference method for the remaining combinations. Overall, 94% of presumptive positive isolates from the test method were confirmed serologically as *E. coli* O157:H7, and 98% of these were also biochemically typical of *E. coli* O157:H7 (completed test). Corresponding rates for the reference method were 69 and 98%, respectively. On the basis of the results of this collaborative study and the precollaborative study that preceded it, it is recommended that this method be adopted official first action for enumeration of *E. coli* O157:H7 in meats, poultry, dairy foods, infant formula, liquid eggs, mayonnaise, and apple cider.

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The recommendation was approved by the Commitee on Microbiology and Extraneous Materials, and was adopted by the Official Methods Board of the Association. *See* "Official Methods Board Actions" (1997) *J. AOAC Int.* **80**, 84A, and "Official Methods Board Actions" (1997) *Inside Laboratory Management*, August issue.

Since the initial outbreak of *Escherichia coli* O157:H7 illnesses in 1982, several methods have been proposed to detect this pathogen in foods. In addition to conventional methods such as the procedures contained in the *Bacteriological Analytical Manual* (1), methods have been developed based on enzyme-labeled immunosorbent assay (ELISA) tests (2–4), "dipstick" immunoassays (5), and immunomagnetic beads (6). These procedures use an enrichment step to maximize sensitivity.

Although most of the emphasis to date has been on testing for the presence or absence of *E. coli* O157:H7, in several situations, an enumeration method can be very useful. For example, when analyzing swab samples from a plant environment, testing water samples, or monitoring raw meats and raw milks, a direct quantitative method would eliminate the need for an enrichment step. Studies to establish the infectious dose of *E. coli* O157:H7 and challenge studies to determine the organism's survival during processing or in the presence of antimicrobial agents also would benefit from a direct enumeration method.

In 1986, Szabo et al. (7) described a new culture medium, HC agar, to be used with membrane filtration for direct enumeration of *E. coli* O157:H7. Their method required 3 sequential readings on the same filter, each reading for a different biochemical reaction. Colonies matching all 3 reaction criteria were considered to be presumptive *E. coli* O157:H7. The final reaction in the series was an in situ indole reaction, which produced total loss of viability. Therefore, it was necessary to subculture colonies matching the first and second reading criteria prior to developing the indole test. After reading the indole reaction, indole-negative isolates were discarded, resulting in a large amount of wasted labor.

To overcome the deficiencies of the Szabo method, we developed a new selective and differential culture

medium, SD-39 agar, for use with the ISO-GRID hydrophobic grid membrane filter. This medium relies on 3 differential biochemical reactions—lysine decarboxylase, sorbitol fermentation, and β-glucuronidase—which are read simultaneously. Selectivity is achieved through use of monensin to inhibit gram-positive bacteria, incubation at 44.0°–44.5°C to inhibit many gram-negative bacteria, and novobiocin to slow the growth of some of the fast-growing temperature-tolerant gram-negative bacteria, such as *Klebsiella* spp. *E. coli* O157:H7 cells grow at this temperature in the presence of NaCl and in the absence of bile salts (7). In addition, $MgSO_4 \cdot 7H_2O$ and sodium glucuronate enhance the repair and growth of injured *E. coli* O157:H7 cells that may be present in the sample. At the specified incubation temperature, presumptive *E. coli* O157:H7 appear pink; other *E. coli* are green, and most other organisms either are unable to initiate growth, or they develop yellow colonies. Confirmation of presumptive positive results is achieved by O157 and H7 serological tests and by verifying pigmentation on tryptone soy agar (TSA). *Hafnia alvei*, a common competitor that would otherwise mimic the appearance of *E. coli* O157:H7 is inhibited at 44.0°–44.5°C. *E. hermanii*, which can cross-react with both O157 and H7 antisera, typically produces a yellow colony on SD-39 agar and yellow pigmentation on TSA.

After the successful completion of a precollaborative study on various food products (8), a collaborative study of this method was performed in 15 government, industry, and university laboratories. This report presents results of the collaborative study.

Collaborative Study

Fifteen laboratories participated in this study, with individual collaborators analyzing from one to 6 food products. Each collaborator received a complete set of instructions, data reporting sheets, and a set of 8 samples for each food product. The Associate Referee also provided each collaborator with positive control cultures (*E. coli*, *E. coli* O157:H7, and *E. hermanii*), radiation-sterilized enzyme powders, enzymatic soaking detergent, SD-39 agar, and sufficient filters for the study. Collaborators not already equipped with filtration units and clamps were provided with a sufficient number for the study. Other materials were furnished by participating laboratories.

Six food products were analyzed: pasteurized apple cider, pasteurized 2% milk, cottage cheese, raw ground beef, cooked ground pork, and frozen whole egg. Each product was inoculated at 3 different concentrations with a pool of 3 isolates of *E. coli* O157:H7 to achieve target inoculum levels of 500/g (high), 200/g (medium), and 50/g (low) on the day testing was initiated. Each inoculated sample also received a pool of competing flora—consisting of equal numbers of 3 isolates each of *E. coli*, *E. hermanii*, and *H. alvei*—at a target concentration of 500/g. Foods were inoculated in bulk at each level, mixed, and then subdivided into individual sample bags. The bags were sealed and either refrigerated (4°–6°C; apple cider, milk, and cheese) or frozen (−18°C; raw beef, cooked pork, and whole egg). Uninoculated products were subdivided into individual samples and stored similarly. Each collaborator received an 8-sample set consisting of a sample pair of uninoculated food product and a blind replicate sample pair of food product from each inoculation level. A replicate 8-sample set from each food product was retained by the originating laboratory for analysis according to the collaborative study schedule.

Frozen whole egg was analyzed by 2 procedures: direct incubation of Hydrophobic Grid Membrane Filter on SD-39 agar (direct test method) and initial incubation on TSA for 4–5 h at 36° ± 1°C before placing filter on SD-39 agar (resuscitation test method). Other foods were analyzed only by the direct test method.

The reference method was based on the procedure for enriching and isolating *E. coli* O157:H7 described in the *Bacteriological Analytical Manual* (1). Culture media and diluents were prepared as described therein. Samples were homogenized in Butterfield's phosphate buffer, and a series of 10-fold dilutions was prepared in the same diluent. A 3-tube, 3-dilution most-probable-number (MPN) series in modified tryptone soy broth (mTSB) was inoculated with triplicate 1 mL volumes of appropriate sample dilutions and incubated at 35°–37°C for 18–24 h. One loopful of liquid was streaked from each tube to a plate of HC agar, which was incubated at 43°C for 18–24 h. Colonies were examined for sorbitol fermentation, and plates containing sorbitol-negative colonies were viewed under long-wave (365 nm) ultraviolet light for glucuronidase production. Two sorbitol- and glucuronidase-negative colonies (presumptive *E. coli* O157:H7) were subcultured from each HC plate to TSA and tryptone soy tryptose broth (TSTB). Confirming tests were performed as described below for the test method. At each dilution, the number of tubes found to contain confirmed *E. coli* O157:H7 was converted to the corresponding MPN index and multiplied by a dilution factor, if appropriate, to obtain the confirmed *E. coli* O157:H7 MPN per gram or milliliter.

Each isolate from both the reference method and the test method also was characterized with several biochemical tests—including indole, methyl red, Voges-ProsKauer, citrate, cellobiose fermentation, sorbitol fermentation, and β-glucuronidase activity—to determine the adequacy of pigmentation on TSA, O157 slide agglutination, and H7 tube agglutination tests as confirming tests for *E. coli* O157:H7. MPN indices from test and reference methods based on these additional tests were reported as completed MPN/g or mL.

997.11, *Escherichia coli* O157:H7 Counts in Foods, Hydrophobic Grid Membrane Filter (ISO-GRID) Method Using SD-39 Agar and Serological Confirmation

First Action 1997

(Applicable to enumeration of *E. coli* O157:H7 from meats, poultry, dairy foods, infant formula, liquid eggs, mayonnaise, and apple cider.)

Method Performance:

See Tables **997.11A** and **B** for method performance data.

A. Principle

Hydrophobic grid membrane filter method uses membrane filter imprinted with hydrophobic material in grid pattern. Hydrophobic lines act as barriers to spread of colonies, thereby dividing membrane filter surface into separate compartments of equal and known size. After incubation, number of squares occupied by colonies is enumerated and converted to most probable number (MPN) value of organisms by using formula.

SD-39 agar contains selective agents to inhibit growth of gram-positive bacteria and some gram-negative bacteria. Elevated incubation temperature also inhibits some gram-negative bacteria. Differential reactions (lysine decarboxylase positive, glucuronidase negative, sorbitol negative) distinguish between presumptive *E. coli* O157:H7 and most other remaining gram-negative bacteria. Confirmation of presumptive positive results is based on absence of yellow colony pigmentation when incubated on tryptone soy agar and on positive agglutination reactions with O157 and H7 antisera.

B. Apparatus, Culture Media, and Reagents

(**a**) *Hydrophobic grid membrane filter (filter).*—Membrane filter has pore size of 0.45 μm and is imprinted with nontoxic hydrophobic material in grid pattern. ISO-GRID (QA Life Sciences, Inc., San Diego, CA), or equivalent, meets these specifications.

(**b**) *Filtration units for hydrophobic grid membrane filter.*—With 5 μm mesh prefilter to remove food particles during filtration. One unit for each sample. ISO-GRID (QA Life Sciences, Inc.), or equivalent, meets these specifications.

(**c**) *Pipettes.*—1.0 mL serological with 0.1 mL graduations, 1.1 or 2.2 mL milk pipettes are satisfactory; 5.0 mL serological with 0.1 mL graduations; 10.0 mL serological with 0.1 mL graduations.

(**d**) *Blender.*—Waring blender, or equivalent, multispeed model with low-speed operation at 10 000–12 000 rpm, and 250 mL glass or metal blender jars with covers. One jar is required for each sample.

(**e**) *Vacuum pump.*—Water aspirator vacuum source is satisfactory.

(**f**) *Manifold or vacuum flask.*

(**g**) *Peptone diluent.*—Dissolve 1.0 g peptone (gelatin hydrolysate peptone; Difco or equivalent) in 1 L water. Dispense enough volume into dilution bottles to give 90 ± 1 mL or 99 ± 1 mL after autoclaving 15 min at 121°C.

(**h**) *Peptone/Tween 80 (PT) diluent.*—Dissolve 1.0 g peptone (gelatin hydrolysate peptone; Difco or equivalent) and 10.0 g Tween 80 in 1 L water. Dispense enough volume into dilution bottles to give 90 ± 1 mL or 99 ± 1 mL after autoclaving 15 min at 121°C.

(**i**) *SD-39 Agar.*—5.0 g proteose peptone, 3.0 g yeast extract, 5.0 g NaCl, 10.0 g L-lysine-HCl, 2.5 g D-glucose, 20.0 g sorbitol, 1.5 g $MgSO_4 \cdot 7H_2O$, 38 mg Na-monensin (Sigma Chemical Co.), 0.5 g Na-glucuronate, 7.5 mg novobiocin, 120 mg phenol red, 50 mg 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt (X-gluc, Biosynth AG), and 15.0 g agar diluted to 1 L with water (SD-39 agar; QA Life Sciences, Inc., or equivalent, is satisfactory). Heat to boiling with stirring until completely dissolved. Do not autoclave. Temper to 45°–50°C. Aseptically pour a sufficient volume of the medium into a small weighing boat or into a Petri dish to produce an agar depth of at least 3 mm. Allow agar to solidify. Check pH by using a flat-surface combination electrode. Adjust pH, if necessary, to 7.2 ± 0.2 by adding sterile 1N NaOH or HCl. Aseptically pour another small portion of medium and check pH as before. Continue adjustments until pH is within specified range. Dispense ca 18–20 mL portions into 15 × 100 mm Petri dishes. Prepared plates may be stored refrigerated for up to 4 weeks if protected from dehydration. Surface-dry plated medium before use by inverting plates partly open for 15–20 min in 35°C incubator.

(**j**) *Motility agar.*—5.0 g tryptose, 5.0 g NaCl, and 3.0 g agar diluted to 1 L with water. Heat to boiling with constant stirring to dissolve completely. Temper to 45°–50°C. Determine pH and adjust, if necessary to final pH of 7.3 ± 0.2 as described above, in (**i**). Dispense 4 mL volumes into 13 × 100 mm test tubes. Sterilize by autoclaving 15 min at 121°C. Allow to solidify in vertical position (i.e., without slanting tubes). Prepared tubes may be stored refrigerated for up to 4 weeks if protected from evaporation.

(**k**) *Tryptone soy agar (TSA).*—15.0 g tryptone, 5.0 g soy peptone, 5.0 g NaCl, and 15.0 g agar diluted to 1 L with water (commercially available dehydrate is satisfactory). Heat to boiling with stirring until agar is completely dissolved. Sterilize by autoclaving 15 min at 121°C. Temper to 45°–50°C in a water bath. Verify pH and adjust, if necessary, to 7.3 ± 0.2 as described above, in (**i**). Dispense 18–20 mL volumes into Petri dishes.

(**l**) *Tryptone soy tryptose broth (TSTB).*—8.5 g tryptone, 10.0 g tryptose, 1.5 g soy peptone, 3.0 g yeast extract, 5.0 g NaCl, 1.25 g anhydrous K_2HPO_4, and 1.75 g D-glucose diluted to 1 L with water. Stir without heating until completely dissolved. Verify pH and ad-

Table 997.11A. Means and precision estimates by food and inoculation level for confirmed *E. coli* O157:H7 enumeration by test and reference methods

Food	Level	Method[a]	Log_{10} mean count/g or mL	Precision estimates[b]					
				r	R	S_r	S_R	RSD_r %	RSD_R %
Apple cider	High	Test	2.57	0.53	0.73	0.19	0.26	7.27	10.10
		Reference	2.05	0.71	2.68	0.25	0.95	12.27	46.17
	Medium	Test	2.32	0.28	0.59	0.10	0.21	4.32	9.03
		Reference	1.95	0.70	2.31	0.25	0.82	12.64	41.81
	Low	Test	1.83	0.44	0.48	0.16	0.17	8.54	9.33
		Reference	1.49	0.89	1.69	0.31	0.60	21.10	40.07
	Overall	Test	2.23						
		Reference	1.83						
Pasteurized milk	High	Test	2.84	1.60	1.76	0.57	0.62	19.89	21.86
		Reference	1.93	1.77	2.80	0.62	0.99	32.41	51.26
	Medium	Test	2.42	1.71	1.58	0.60	0.56	24.98	23.15
		Reference	1.58	0.89	1.92	0.31	0.68	19.83	42.88
	Low	Test	2.15	1.23	1.13	0.43	0.40	20.19	18.63
		Reference	1.24	1.54	2.05	0.54	0.73	43.97	58.74
	Overall[c]	Test	2.46						
		Reference	1.58						
Cottage cheese	High	Test	2.35	1.12	1.24	0.39	0.44	16.82	18.66
		Reference	2.10	1.22	2.68	0.43	0.95	20.65	45.28
	Medium	Test	2.15	1.44	1.87	0.51	0.66	23.62	30.70
		Reference	1.77	0.99	2.38	0.35	0.84	19.73	47.58
	Low	Test	1.82	0.83	1.83	0.29	0.65	16.02	35.41
		Reference	1.28	0.99	2.03	0.35	0.72	27.40	56.23
	Overall	Test	2.11						
		Reference	1.71						
Cooked ground pork	High	Test	2.04	1.57	1.63	0.55	0.58	27.14	28.18
		Reference	1.44	2.21	2.52	0.78	0.89	54.04	61.84
	Medium	Test	1.79	0.73	1.18	0.26	0.42	14.35	23.20
		Reference	1.41	0.69	2.22	0.24	0.78	17.26	55.46
	Low	Test	1.12	0.49	0.59	0.17	0.21	15.51	18.63
		Reference	0.83	0.49	1.70	0.17	0.60	20.89	72.50
	Overall[c]	Test	1.65						
		Reference	1.24						
Raw ground beef	High	Test	2.29	0.81	1.06	0.29	0.37	12.55	16.32
		Reference	1.18	1.50	2.01	0.53	0.71	44.84	59.98
	Medium	Test	1.64	1.13	0.96	0.40	0.34	24.36	20.71
		Reference	0.99	1.31	1.33	0.46	0.47	46.91	47.52
	Low	Test	1.12	0.46	0.50	0.16	0.18	14.61	15.90
		Reference	0.61	0.55	0.72	0.19	0.25	31.98	41.87
	Overall[c]	Test	1.73						
		Reference	0.93						
Frozen whole egg	High	Direct	2.95	0.55	1.24	0.19	0.44	6.54	14.83
		Resuscitation	3.20	0.77	1.21	0.27	0.43	8.54	13.40
		Reference	2.72	1.78	2.99	0.63	1.06	23.13	38.83
	Medium	Direct	1.12	0.50	0.84	0.18	0.30	15.70	26.52
		Resuscitation	1.25	0.66	0.98	0.23	0.35	18.65	27.71
		Reference	1.03	1.04	1.47	0.37	0.52	35.66	50.43
	Low	Direct	1.19	1.51	1.45	0.54	0.51	44.87	42.85
		Resuscitation	1.16	0.50	0.91	0.18	0.32	15.19	27.64
		Reference	0.83	1.20	1.32	0.42	0.47	50.96	55.93
	Overall[d]	Direct	1.76						
		Resuscitation	1.87						
		Reference	1.53						

[a] Test = test method; Reference = reference method; Direct = direct test method; Resuscitation = resuscitation test method.

[b] Precision estimates were calculated after conversion of data to log_{10}. $r = 2.8 \times S_r$; $R = 2.8 \times S_R$; S_r = repeatability standard deviation; S_R = reproducibility standard deviation; RSD_r = repeatability relative standard deviation; RSD_R = reproducibility relative standard deviation.

[c] Difference between methods is significantly different at the 5% level.

[d] Difference between resuscitation test method and reference method is significantly different at the 5% level.

just, if necessary, to 7.2 ± 0.2. Dispense into bottles or tubes as required. Sterilize by autoclaving 15 min at 121°C.

(**m**) *Papain stock solution.*—Reconstitute 10.0 g presterilized papain powder (EZ-Papain; QA Life Sciences, Inc.) with 100 mL sterile water. Unused portion can be stored frozen for up to 3 months.

(**n**) *Cellulase stock solution.*—Reconstitute 20.0 g presterilized cellulase powder (EZ-Cellulase; QA Life Sciences, Inc.) with 100 mL sterile water. Unused portion can be stored frozen for up to 3 months.

(**o**) *Amyloglucosidase stock solution.*—Reconstitute 20.0 g presterilized amyloglucosidase powder (EZ-AMG; QA Life Sciences, Inc.) with 100 mL sterile water. Unused portion can be stored frozen for up to 3 months.

(**p**) *Alkaline protease stock solution.*—Reconstitute 20.0 g presterilized alkaline protease powder (EZ-APUG; QA Life Sciences, Inc.) with 100 mL sterile water. Unused portion can be stored frozen for up to 3 months.

(**q**) *E. coli O157 antiserum.*

(**r**) *E. coli H7 antiserum.*

(**s**) *Sterile glass shot.*

C. Sample Preparation

(**a**) *Liquid egg.*—Thoroughly mix sample with sterile spoon or spatula. Prepare 1:10 dilution by aseptically weighing 11 g egg material into sterile wide-mouth, screw-top bottle; add 99 mL PT diluent, **B(h)**, and 1 tablespoonful of sterile glass shot. Thoroughly agitate 1:10 dilution to ensure complete solution or distribution of egg material in diluent by shaking each bottle rapidly 25 times through a 30 cm arc within ≤7s. Let bubbles escape. If enzyme treatment is needed (Table **997.11B**), combine 5 mL 1:10 dilution with 1 mL appropriate enzyme stock solution and mix by pipeting up and down or by shaking gently. Incubate solution 20–30 min in 35°–37°C water bath. Correct for additional dilution by filtering 1.2 mL enzyme-treated sample.

(**b**) *Other liquid samples.*—Thoroughly mix contents of sample container. To prepare 1:10 dilution, aseptically transfer 10 mL sample into 90 mL peptone diluent, **B(g)**, or PT diluent, **B(h)** (Table **997.11B**), in sterile wide-mouth, screw-top bottle. Mix by shaking bottle 25 times through 30 cm arc in ≤7s. If enzyme treatment is needed (Table **997.11B**), combine 5 mL 1:10 dilution with 1 mL appropriate enzyme stock solution and mix by pipeting up and down or by shaking gently. Incubate solution 20–30 min in 35°–37°C water bath. Correct for additional dilution by filtering 1.2 mL enzyme-treated sample.

(**c**) *Powdered foods.*—Thoroughly mix sample with sterile spoon or spatula. Prepare 1:10 dilution by aseptically weighing 10 g sample into sterile wide-mouth, screw-top bottle; add 90 mL peptone, **B(g)**, or PT diluent, **B(h)** (Table **997.11B**), and shake bottle rapidly 25 times through 30 cm arc in ≤7s. Let bubbles escape.

Table 997.11B. Diluents and enzyme treatments for foods[a]

Food	Diluent	Enzyme
Raw ground beef	PT[b]	None
Cooked ground beef	PT	None
Raw ground turkey meat	PT	None
Cooked ground turkey meat	PT	None
Raw ground pork	PT	None
Cooked ground pork	PT	None
Raw ground lamb	PT	None
Raw fermented sausage	PT	None
Raw milk	PT	Papain
Pasteurized milk	PT	Papain
Ice cream	Peptone[c]	Papain + AMG[d]
Cottage cheese	PT	Papain
Cheddar cheese	PT	Papain
Cream cheese	PT	Papain
Liquid infant formula	Peptone	Papain
Apple cider	Peptone	None
Pasteurized whole egg	Peptone	APUG[e]
Mayonnaise	Peptone	Papain + AMG
Dry infant formula	Peptone	Papain + cellulase

[a] Based on analysis of 1 mL of a 1:10 dilution. Foods tested at dilutions of 1:100 or higher do not usually need enzyme treatment. Also refer to AOAC Methods **986.32** and **995.21** for recommended enzyme treatments of foods not listed in this table.

[b] PT, 0.1% peptone + 1.0% Tween 80 diluent.

[c] Peptone, 0.1% peptone diluent.

[d] AMG, amyloglucosidase enzyme.

[e] APUG, alkaline protease enzyme.

If enzyme treatment is needed (Table **997.11B**), combine 5 mL 1:10 dilution with 1 mL appropriate enzyme stock solution and mix by pipeting up and down or by shaking gently. Incubate solution 20–30 min in 35°–37°C water bath. Correct for additional dilution by filtering 1.2 mL enzyme-treated sample.

(d) *Other foods.*—To prepare 1:10 dilution, aseptically weigh 10 g sample into sterile blender jar. Add 90 mL peptone, **B(g)**, or PT diluent, **B(h)** (Table **997.11B**), and blend 2 min at low speed (10 000–12 000 rpm). If enzyme treatment is needed (Table **997.11B**), combine 5 mL 1:10 dilution with 1 mL appropriate enzyme stock solution and mix by pipeting up and down or by shaking gently. Incubate solution 20–30 min in 35°–37°C water bath. Correct for additional dilution by filtering 1.2 mL enzyme-treated sample.

D. Analysis

(*See* Figs. **986.32A** and **986.32B**.) Turn on vacuum source. Place sterile filtration unit on manifold or vacuum flask. Open clamp A. Rotate back funnel portion C. Aseptically place sterile filter, **B(a)**, on surface of base D. Rotate funnel forward. Clamp shut by sliding jaws L of stainless steel clamp over entire length of flanges B that extend from both sides of funnel C and base D and rotating moving arm K into horizontal (locked) position.

Aseptically add ca 15–20 mL sterile water to funnel. Pipette 1.0 mL 1:10 dilution (or appropriate volume of enzyme-treated sample) into funnel. Apply free end of vacuum tubing E to suction hole F to draw liquid through prefilter mesh G. Aseptically add additional 10–15 mL sterile water to funnel and draw through mesh as before. Close clamp A to direct vacuum to base of filtration unit and draw liquid through filter.

Open clamp A. Rotate moving arm K of stainless steel clamp into unlocked (ca 45° angle) position and slide jaws L off flanges B. Rotate back funnel C.

(a) *Presumptive result.*—Place filter on surface of SD-39 agar plate. Incubate 24 ± 2 h at 44.0°–44.5°C. Do not use an ultraviolet lamp to examine filter. Examine filter in natural light, under incandescent lighting, or under fluorescent lighting for presence of orange or pink colonies (presumptive positive *E. coli* O157:H7). Other organisms will produce green or yellow colonies. Occasionally, a mixed square containing both a pink and a green colony will appear purple. This should also be considered presumptive positive.

Add the number of squares containing one or more presumptive positive colonies as described above. The total is the presumptive score.

(b) *Confirmed result.*—Select up to 5 presumptive positive squares and subculture to TSA and TSTB. Incubate 18–24 h at 36° ± 1°C. Examine TSA for purity and purify, if necessary, by inoculating fresh TSA and TSTB with an isolated colony and incubating as described above. Examine purified cultures for pigmentation and discard any pigmented isolates without carrying out serological testing. If all 5 isolates are pigmented, report as less than {reciprocal of dilution factor} *E. coli* O157:H7/g or mL.

Perform slide agglutination test on nonpigmented isolates as follows: (*1*) mark off 2 areas on a clean glass microscope slide with a wax pencil, (*2*) deposit a drop of sterile saline into each area on the slide, (*3*) emulsify material from TSA culture in both drops of saline (use enough material to make a uniform turbid emulsion), (*4*) add one drop of O157 antiserum to one of the culture drops, and (*5*) rock slide back and forth for ca 1 min and watch for agglutination to occur. Agglutination should be recorded as positive only if the control drop remains smoothly emulsified.

Record agglutination result and discard isolates that do not react in slide agglutination test with O157 antiserum. If all nonpigmented isolates also are not reactive to O157 antiserum, report as less than {reciprocal of dilution factor} *E. coli* O157:H7/g or mL.

Perform H7 tube agglutination test on nonpigmented, O157 positive isolates as follows (always include a known positive culture with each series of tube agglutination tests to confirm that reagents are functioning correctly): (*1*) Inactivate 1 mL TSTB culture by adding 20 μL 37% formaldehyde (commercial full-strength solution). If a 20 μL pipettor is not available, prepare a 1/5 dilution of the 37% formaldehyde in saline and transfer 0.1 mL diluted formaldehyde into the TSTB culture. (*2*) Dilute H7 antiserum in saline according to manufacturer's directions. Transfer 0.5 mL diluted H7 antiserum into a sterile 13 × 100 mm glass culture tube. (*3*) Add 0.5 mL inactivated TSTB culture (*1*) to diluted antiserum (*2*). Shake tube gently to mix. (*4*) Add 0.5 mL saline to remaining 0.5 mL inactivated TSTB culture (*1*) as a negative control. Shake tube gently to mix. (*5*) Incubate both tubes in water bath at 50° ± 1°C for 60–90 min. (*6*) Carefully remove tubes from water bath after 60–90 min have elapsed. Without shaking tubes or disturbing contents, examine for evidence of agglutination in tube containing H7 antiserum. If agglutination is present, record as positive result.

If H7 agglutination test is negative, induce motility as follows: (*1*) Inoculate a tube of motility agar by stabbing in the center of the tube. Incubate 18–24 h at 35°C. (*2*) Examine for evidence of motility. Motile cultures will grow in a diffuse pattern away from the area of the stab, both within the agar and over the surface of the agar. Nonmotile cultures will grow only in the immediate vicinity of the stab. (*3*) Subculture motile cultures into fresh TSTB medium. Incubate 18–24 h at 35°C, and repeat H7 agglutination procedure. (*4*) Reinoculate nonmotile cultures into fresh motility agar. Incubate and examine as in (*1*) and (*2*).

If culture is still nonmotile after 3 attempts to induce motility, record as nonmotile for the H7 result. If culture is motile but the H7 agglutination test is still

negative after 3 attempts, record as a negative H7 result.

Isolates that are unpigmented on TSA and give a positive reaction to both O157 and H7 antisera are confirmed as *E. coli* O157:H7. Determine the proportion of the original 5 isolates that meet these criteria (e.g., 3/5) and multiply this fraction by the presumptive score to obtain the confirmed score. Convert the confirmed score to an MPN index:

$$\text{MPN} = 1600 \times \log_e \frac{1600}{1600 - x}$$

where x = number of positive squares. Multiply MPN by reciprocal of dilution factor, round to 2 significant figures, and report as *E. coli* O157:H7/g or mL.

Isolates that are unpigmented on TSA, give a positive reaction to O157 antiserum, and are nonmotile are confirmed as *E. coli* O157:NM. Determine the proportion of the original 5 isolates that meet these criteria and multiply this fraction by the presumptive score to obtain the confirmed score. Convert the confirmed score to an MPN index as described above.

Multiply MPN by reciprocal of dilution factor, round to 2 significant figures, and report as *E. coli* O157:NM/g or mL.

Ref.: *J. AOAC Int.* **81**, 403 (1998).

Results and Discussion

Reported results were checked for correct determination of presumptive, confirmed, and completed MPN indices for both reference and test methods; for correct calculation of confirmed and completed counts based on serological and biochemical results; and for correct choice of dilution factors. Results were rounded to 2 significant figures and converted to $\log_{10}$ for statistical analysis. The confirmed test and reference method data are reported in Tables 1–6.

Data exclusions due to significant method deviations or statistical outliers are described below for each food. For data exclusions affecting all 8 samples of a food analyzed by a collaborator, results from both test and reference methods were excluded from statistical evaluation even if the method deviation or statistical outlier involved only one of the methods.

Apple Cider

Results are reported in Tables 1A and 1B. Collaborator 1 deviated significantly from both test and reference methods by reporting biochemical and serological tests results on an inadequate number of isolates. Collaborator 14 experienced a temperature control problem with the test method incubator. Data from both collaborators were excluded from statistical analysis. In addition, test method results of collaborator 5 for the high inoculation level were outliers as determined by the Cochran outlier screening method (9) and were excluded from calculations of test method performance. The test method produced numerically higher counts at all 3 inoculum levels, but these did not differ

Table 1A. Collaborative study results for confirmed *E. coli* O157:H7 counts in apple cider by test method

	E. coli O157:H7, count/mL							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
1[b]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	N/A[c]	N/A	N/A	N/A	N/A	N/A
2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	5.0×10^1	8.0×10^1	2.7×10^2	2.3×10^2	5.6×10^2	4.1×10^2
3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	6.0×10^1	5.0×10^1	2.4×10^2	1.7×10^2	3.8×10^2	8.0×10^1
4	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.2×10^2	7.0×10^1	1.8×10^2	2.5×10^2	2.7×10^2	2.4×10^2
5[d]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^1	1.0×10^2	2.5×10^2	2.6×10^2	6.5×10^2	2.2×10^1
7	$<1.0 \times 10^1$	$<1.0 \times 10^1$	7.0×10^1	6.4×10^1	3.3×10^2	4.1×10^2	4.4×10^2	4.2×10^2
9	$<1.0 \times 10^1$	$<1.0 \times 10^1$	5.0×10^1	1.0×10^2	3.4×10^2	2.2×10^2	5.6×10^2	5.1×10^2
11	$<1.0 \times 10^1$	$<1.0 \times 10^1$	5.0×10^1	3.0×10^1	8.0×10^1	1.5×10^2	3.0×10^2	1.7×10^2
13	$<1.0 \times 10^1$	$<1.0 \times 10^1$	7.0×10^1	7.0×10^1	9.0×10^1	9.0×10^1	3.7×10^2	3.9×10^2
14[e]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.7×10^1	1.4×10^2	$<1.0 \times 10^1$	1.2×10^2	8.4×10^1	2.7×10^2
15	$<1.0 \times 10^1$	$<1.0 \times 10^1$	9.0×10^1	1.4×10^2	3.0×10^2	2.3×10^2	7.3×10^2	9.7×10^2

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

[b] Significant method deviation in test and reference methods. Data were excluded from statistical analysis.

[c] N/A, not available.

[d] Test method results for high inoculation level were outliers as determined by the Cochran outlier screen and were excluded from calculations of test method performance.

[e] Collaborator reported incorrect incubator temperature for test method. Data from test and reference methods were excluded from statistical analysis.

Table 1B. Collaborative study results for confirmed *E. coli* O157:H7 counts in apple cider by reference method

	E. coli O157:H7, count/mL							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
1[b]	$<3.0 \times 10^0$	$<3 \times 10^0$	N/A[c]	N/A	N/A	N/A	N/A	N/A
2	$<3.0 \times 10^0$	$<3 \times 10^0$	9.3×10^1	1.5×10^2	2.3×10^2	9.3×10^2	4.3×10^2	9.3×10^2
3	$<3.0 \times 10^0$	$<3 \times 10^0$	4.3×10^2	7.5×10^1	2.1×10^2	7.5×10^1	4.3×10^2	4.3×10^2
4	$<3.0 \times 10^0$	$<3 \times 10^0$	4.3×10^1	9.3×10^1	4.3×10^2	9.3×10^2	9.3×10^1	2.1×10^2
5	$<3.0 \times 10^0$	$<3 \times 10^0$	1.1×10^1	1.1×10^1	4.3×10^1	2.9×10^1	1.1×10^1	1.3×10^1
7	$<3.0 \times 10^0$	$<3 \times 10^0$	3.9×10^1	9.3×10^1	2.3×10^2	2.3×10^2	2.3×10^2	9.3×10^2
9	$<3.0 \times 10^0$	$<3 \times 10^0$	1.5×10^1	2.1×10^1	3.4×10^1	1.1×10^1	5.3×10^1	1.5×10^2
11	$<3.0 \times 10^0$	$<3 \times 10^0$	6.1×10^0	$<3.0 \times 10^0$	3.0×10^0	$<3.0 \times 10^0$	6.0×10^0	$<3.0 \times 10^0$
13	$<3.0 \times 10^0$	$<3 \times 10^0$	$<3.0 \times 10^0$	2.3×10^1	2.8×10^1	4.3×10^1	1.4×10^1	1.5×10^1
14[d]	$<3.0 \times 10^0$	$<3 \times 10^0$	7.3×10^0	2.1×10^1	$<3.0 \times 10^0$	3.5×10^1	$<3.0 \times 10^0$	2.3×10^2
15	$<3.0 \times 10^0$	$<3 \times 10^0$	4.3×10^1	4.3×10^1	9.3×10^2	4.3×10^2	1.5×10^3	4.6×10^3

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

[b] Significant method deviation in test and reference methods. Data were excluded from statistical analysis.

[c] N/A, not available.

[d] Collaborator reported incorrect incubator temperature for test method. Data from test and reference methods were excluded from statistical analysis.

Table 2A. Collaborative study results for confirmed *E. coli* O157:H7 counts in pasteurized milk by test method

	E. coli O157:H7, count/mL							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
1[b]	N/A[c]	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.7×10^2	1.3×10^2	3.1×10^2	4.7×10^2	8.3×10^2	8.6×10^2
4	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.8×10^2	1.7×10^2	3.6×10^2	3.2×10^2	7.4×10^2	8.2×10^2
5	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.7×10^2	9.2×10^1	3.4×10^2	1.5×10^2	9.8×10^2	1.5×10^3
7[d]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.5×10^4	6.9×10^4	7.0×10^4	6.2×10^4	$<1.0 \times 10^1$	6.2×10^4
8[e]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.8×10^2	$<1.0 \times 10^1$	6.7×10^2	2.1×10^3	1.6×10^3
9	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.5×10^2	2.3×10^2	6.1×10^2	8.2×10^2	1.3×10^3	1.3×10^3
11	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.8×10^2	2.0×10^1	1.9×10^2	1.6×10^2	7.2×10^1	4.5×10^2
12[f]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.7×10^2	1.3×10^2	$<1.0 \times 10^1$	5.3×10^2	$<1.0 \times 10^1$	1.2×10^3
13	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.8×10^2	1.1×10^2	3.4×10^2	3.9×10^2	5.7×10^2	9.4×10^2
14[g]	1.5×10^2	3.0×10^3	2.8×10^2	5.5×10^2	$<1.0 \times 10^1$	2.9×10^2	$<1.0 \times 10^1$	1.6×10^2
15	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.9×10^2	3.4×10^2	5.4×10^2	1.0×10^3	3.7×10^3	2.0×10^3

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

[b] Significant method deviation in test and reference methods. Incomplete data reported.

[c] N/A, not available.

[d] Statistical outlier for test method. Data from test and reference methods were excluded from statistical analysis.

[e] Test method results for low inoculation level were outliers as determined by the Cochran outlier screen and were excluded from calculations of test method performance.

[f] Test method results for high inoculation level were outliers as determined by the Cochran outlier screen and were excluded from calculations of test method performance.

[g] Target organism was isolated from uninoculated control samples by test and reference methods. Data were excluded from statistical analysis.

Table 2B. Collaborative study results for confirmed *E. coli* O157:H7 counts in pasteurized milk by reference method

	E. coli O157:H7, count/mL							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
1[b]	N/A[c]	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	$<3.0 \times 10^0$	$<3.0 \times 10^0$	4.3×10^1	2.3×10^1	9.3×10^1	2.9×10^1	1.6×10^2	2.3×10^2
4	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.1×10^0	4.3×10^1	1.5×10^1	9.1×10^0	1.4×10^1
5	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.6×10^0	2.0×10^1	1.5×10^1	2.0×10^1	9.3×10^0
7[d]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.9×10^2	1.6×10^2	5.3×10^2	2.0×10^2	2.9×10^2	2.9×10^2
8[e]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	4.3×10^2	9.4×10^0	9.4×10^0	9.2×10^0	2.3×10^2
9	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.1×10^2	1.5×10^2	2.8×10^2	2.1×10^2	4.6×10^3	2.4×10^2
11	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.0×10^0	3.0×10^0	$<3.0 \times 10^0$	9.0×10^0	3.0×10^0
12	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.5×10^2	9.3×10^1	2.1×10^2	4.3×10^2	2.0×10^2	9.3×10^2
13[f]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.3×10^1	7.3×10^0	2.3×10^2	2.0×10^1	2.3×10^2	1.3×10^1
14[g]	9.3×10^1	2.1×10^3	2.3×10^2	7.5×10^2	$<3.0 \times 10^0$	4.3×10^2	$<3.0 \times 10^0$	2.1×10^2
15	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.1×10^0	1.5×10^1	4.3×10^1	7.5×10^1	4.3×10^2	4.6×10^3

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

[b] Significant method deviation in test and reference methods. Incomplete data reported.

[c] N/A, not available.

[d] Statistical outlier for test method. Data from test and reference methods were excluded from statistical analysis.

[e] Reference method results for low inoculation level were outliers as determined by the Cochran outlier screen and were excluded from calculations of reference method performance.

[f] Reference method results for medium inoculation level were outliers as determined by the Cochran outlier screen and were excluded from calculations of reference method performance.

[g] Target organism was isolated from uninoculated control samples by test and reference methods. Data were excluded from statistical analysis.

significantly from results obtained by the reference method (probability [Pr] > F of 0.0911). Repeatability and reproducibility values by the test method were consistently lower than those by the reference method (*see* Table **997.11A**).

Pasteurized Milk

Results are reported in Tables 2A and 2B. Collaborator 1 deviated significantly from both test and reference methods (*see* description of error in *Apple Cider*).

Table 3A. Collaborative study results for confirmed *E. coli* O157:H7 counts in cottage cheese by test method

	E. coli O157:H7, count/g							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
4	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.0×10^1	8.0×10^1	1.3×10^2	3.1×10^2	1.1×10^2
5[b]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^1	1.0×10^1	3.1×10^3	4.0×10^1	5.6×10^2	7.0×10^1
7	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.9×10^2	2.3×10^3	5.8×10^2	3.6×10^2	3.6×10^2	3.3×10^2
8	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^1	4.0×10^1	1.0×10^1	8.0×10^1	$<1.0 \times 10^1$	2.4×10^2
9	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.0×10^2	3.8×10^2	2.2×10^2	2.4×10^2	3.1×10^2	2.6×10^2
11	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^1	1.0×10^1	2.0×10^1	1.0×10^1	8.0×10^1	1.2×10^2
12	$<1.0 \times 10^1$	$<1.0 \times 10^1$	8.0×10^1	1.7×10^2	4.1×10^2	6.4×10^2	5.2×10^2	5.2×10^2
13	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.0×10^1	4.0×10^1	6.0×10^1	1.2×10^2	2.1×10^2	1.8×10^2
14	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.7×10^2	2.3×10^2	2.9×10^2	1.2×10^3	1.1×10^3	4.9×10^2
15	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^1	4.0×10^1	6.0×10^1	1.2×10^2	2.8×10^2	1.9×10^2

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

[b] Test method results for medium inoculation level were outliers as determined by the Cochran outlier screen and were excluded from calculations of test method performance.

Table 3B. Collaborative study results for confirmed *E. coli* O157:H7 counts in cottage cheese by reference method

	E. coli O157:H7, count/g							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
4	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.3×10^1	4.3×10^1	1.5×10^2	3.9×10^2	1.2×10^2	7.5×10^2
5	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	4.3×10^1	6.1×10^0	2.1×10^1	9.3×10^1
7	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.0×10^0	6.1×10^0	6.0×10^0	$<3.0 \times 10^0$	3.0×10^0
8	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.9×10^1	2.1×10^2	9.3×10^1	2.3×10^2	2.9×10^2	4.6×10^3
9	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.5×10^1	3.0×10^0	1.6×10^1	2.9×10^1	2.3×10^2	4.3×10^2
11	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.0×10^0	6.0×10^0	$<3.0 \times 10^0$
12	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.5×10^1	9.1×10^0	2.3×10^2	9.3×10^2	2.3×10^2	4.3×10^2
13	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.3×10^1	2.3×10^1	1.5×10^2	1.5×10^2	4.3×10^2	4.3×10^2
14	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.3×10^2	4.3×10^2	2.3×10^2	2.1×10^3	1.2×10^3	4.4×10^2
15	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.5×10^1	9.3×10^1	5.3×10^1	3.6×10^1	4.3×10^2	5.3×10^1

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

Collaborator 7's test method data were extreme outliers, being more than 2 $\log_{10}$ cycles higher than the next closest set of data for most of the samples. Collaborator 14 isolated *E. coli* O157:H7 from uninoculated control samples by both methods. Data from collaborators 1, 7, and 14 were excluded from statistical analysis. Test method results of collaborator 8 for the low inoculation level and of collaborator 12 for the high inoculation level were outliers as determined by the Cochran outlier screen and were excluded from calculations of test method performance. Reference method results of collaborator 8 for the low inoculation level and of collaborator 13 for the medium inoculation level were outliers as determined by the Cochran outlier screen and were excluded from calculations of reference method performance. The test method produced confirmed *E. coli* O157:H7 results that were significantly higher than those of the reference method ($Pr > F$ of 0.0039). Repeatability values were similar for both methods, but test method reproducibility values were consistently lower than reference method reproducibility values for all 3 inoculation levels (*see* Table **997.11A**).

Cottage Cheese

Results are reported in Tables 3A and 3B. No deviations from methods were reported. Test method results of collaborator 5 for the medium inoculation level were outliers as determined by the Cochran outlier screen and were excluded from calculations of test method performance. The test method produced numerically higher counts than the reference method at all 3 inoculum levels, but the differences were not significant

Table 4A. Collaborative study results for confirmed *E. coli* O157:H7 counts in cooked ground pork by test method

	E. coli O157:H7, count/g							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
4	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.0×10^1	$<1.0 \times 10^1$	5.0×10^1	1.6×10^2	4.2×10^2	4.1×10^2
5	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	6.0×10^1	1.2×10^2	4.0×10^1
7	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^1$	4.8×10^1	5.6×10^1	1.0×10^1	3.4×10^2
8	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^1	3.0×10^1	1.8×10^2	1.2×10^2	$<1.0 \times 10^1$	3.9×10^2
11	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^1	1.0×10^1	1.4×10^2	6.0×10^1	8.0×10^1	1.0×10^2
12	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^1$	8.0×10^1	1.0×10^2	1.4×10^2	2.3×10^2
13	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^1	$<1.0 \times 10^1$	9.0×10^1	7.2×10^1	2.8×10^2	2.4×10^2
14	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^1
15	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1	1.0×10^1	1.9×10^2	8.0×10^1	2.5×10^2	3.3×10^2

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

Table 4B. Collaborative study results for confirmed *E. coli* O157:H7 counts in cooked ground pork by reference method

	E. coli O157:H7, count/g							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
4	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.6×10^0	3.6×10^0	2.3×10^2	1.5×10^2	2.3×10^2	4.3×10^1
5	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.1×10^0	3.6×10^0	7.3×10^0
7	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.6×10^0	3.6×10^0	$<3.0 \times 10^0$	9.1×10^0	9.1×10^0	2.3×10^1
8[b]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.3×10^1	2.3×10^2	2.3×10^2	2.3×10^2	$<3.0 \times 10^0$	1.5×10^3
11	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.0×10^0	9.1×10^0	9.3×10^0	9.3×10^0
12	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.1×10^0	3.6×10^0	2.3×10^2	9.3×10^1	2.3×10^2	4.3×10^2
13	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.3×10^1	9.1×10^0	9.3×10^1	4.3×10^1	1.5×10^1	4.3×10^1
14	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	7.3×10^0
15	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.6×10^0	3.6×10^0	4.3×10^1	2.3×10^1	9.3×10^2	4.4×10^1

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

[b] Reference method results for high and low inoculation levels were outliers as determined by the Cochran outlier screen and Grubbs extreme value (mean) test, respectively, and were excluded from calculations of reference method performance.

($Pr > F$ of 0.1780). Repeatability values were similar for both methods, but test method reproducibility values were consistently lower than reference method reproducibility values for all 3 inoculation levels (*see* Table **997.11A**).

Cooked Ground Pork

Results are reported in Tables 4A and 4B. No deviations from methods were reported. Reference method results of collaborator 8 for the high inoculation level

Table 5A. Collaborative study results for confirmed *E. coli* O157:H7 counts in raw ground beef by test method

	E. coli O157:H7, count/g							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
4	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.0×10^1	2.0×10^1	8.0×10^1	4.0×10^1	4.3×10^2	1.5×10^2
5	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^1$	3.0×10^1	7.0×10^1	1.1×10^2	6.0×10^1
6[b]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
7[c]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	6.0×10^1	6.0×10^1	$<1.0 \times 10^1$	1.8×10^2	2.7×10^2
8	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1	3.0×10^1	5.0×10^1	1.0×10^2	8.9×10^2	2.9×10^2
10	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1	8.0×10^1	$<1.0 \times 10^1$	2.5×10^2	2.0×10^2
12[d]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	8.0×10^1	9.6×10^2	2.0×10^2
13	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.0×10^1	$<1.0 \times 10^1$	5.0×10^1	5.0×10^1	1.4×10^2	4.0×10^1
14[e]	$<1.0 \times 10^1$	8.0×10^1	7.0×10^1	2.6×10^2	$<1.0 \times 10^1$	1.0×10^2	1.0×10^2	7.0×10^1
15	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.0×10^1	2.0×10^1	7.0×10^1	8.0×10^1	1.2×10^2	1.1×10^2

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

[b] Samples were exposed to incorrect storage temperature. Data from test and reference methods were excluded from statistical analysis.

[c] Test method results for low inoculation level were outliers as determined by the Cochran outlier screen and were excluded from calculations of test method performance.

[d] Test method results for low inoculation level were outliers as determined by the Grubbs extreme value test and were excluded from calculations of test method performance.

[e] Target organism was isolated from uninoculated control sample by test and reference methods. Data were excluded from statistical analysis.

Table 5B. Collaborative study results for confirmed *E. coli* O157:H7 counts in raw ground beef by reference method

	E. coli O157:H7, count/g							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
4[b]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.1×10^1	3.6×10^0	9.3×10^1	9.1×10^0	5.3×10^1	2.3×10^1
5	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.1×10^0	3.6×10^0	3.0×10^0	3.0×10^0
6[c]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.0×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$
7	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.0×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
8	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.0×10^0	$<3.0 \times 10^0$	3.6×10^0	9.1×10^0	6.1×10^0	2.3×10^1
10	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.0×10^0	1.5×10^1	3.0×10^0	2.3×10^1	1.5×10^1
12	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.6×10^0	3.0×10^0	1.6×10^1	2.0×10^1	4.3×10^2	1.6×10^1
13	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.1×10^0	9.1×10^0	3.6×10^0	4.3×10^1	9.1×10^0	$<3.0 \times 10^0$
14[d]	$<3.0 \times 10^0$	4.3×10^1	2.9×10^2	1.1×10^4	$<3.0 \times 10^0$	1.2×10^2	9.3×10^1	3.9×10^2
15	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.6×10^0	9.1×10^0	4.3×10^1	4.3×10^2	2.1×10^1

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

[b] Reference method results for low inoculation level were outliers as determined by the Cochran outlier screen and were excluded from calculations of reference method performance.

[c] Samples were exposed to incorrect storage temperature. Data from test and reference methods were excluded from statistical analysis.

[d] Target organism was isolated from uninoculated control sample by test and reference methods. Data were excluded from statistical analysis.

were outliers as determined by the Cochran outlier screen; reference method results of this collaborator for the low inoculation level also were outliers as determined by the Grubbs extreme value (mean) test (9). These data were excluded from calculations of reference method performance. Confirmed *E. coli* O157:H7 count results were significantly higher by the test method than by the reference method ($Pr > F$ of 0.0221). Repeatability values were similar for both methods, but test method reproducibility values were consistently lower than reference method reproducibility values for all 3 inoculation levels (*see* Table **997.10A**).

Table 6A. Collaborative study results for confirmed *E. coli* O157:H7 counts in frozen whole egg by direct test method

	E. coli O157:H7, count/g							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.3×10^3	1.1×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
4	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.1×10^3	1.2×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^1$
5	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.3×10^3	1.4×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
6	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.1×10^2	7.7×10^2	9.3×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
7	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.7×10^3	3.3×10^3	1.0×10^1	5.4×10^1	7.2×10^1	5.6×10^1
8[b]	N/A[c]	N/A	N/A	N/A	N/A	N/A	N/A	N/A
9[b]	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
10	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.4×10^3	3.6×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
12	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.5×10^3	1.1×10^3	1.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
13	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.9×10^2	1.7×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
14	$<1.0 \times 10^1$	$<1.0 \times 10^1$	9.0×10^1	8.0×10^1	1.4×10^2	$<1.0 \times 10^1$	6.0×10^1	$<1.0 \times 10^1$
15	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.3×10^3	1.3×10^3	1.0×10^1	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^1$

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

[b] Collaborator did not perform direct test method. Data were excluded from statistical analysis of direct test method vs reference method and of direct test method vs resuscitation test method.

[c] N/A, not available.

Table 6B. Collaborative study results for confirmed *E. coli* O157:H7 counts in frozen whole egg resuscitation test method

	E. coli O157:H7, count/g							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.8×10^3	2.4×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1
4	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.8×10^3	1.5×10^3	$<1.0 \times 10^1$	1.0×10^1	4.0×10^1	$<1.0 \times 10^1$
5	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.8×10^3	2.3×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
6	$<1.0 \times 10^1$	$<1.0 \times 10^1$	7.0×10^1	1.0×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^1	$<1.0 \times 10^1$
7	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.9×10^3	4.3×10^3	2.0×10^1	6.0×10^1	7.2×10^1	6.4×10^1
8[b]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.6×10^3	1.1×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
9[b]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.4×10^3	2.7×10^3	2.0×10^1	2.0×10^1	5.0×10^1	6.0×10^1
10	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^3	2.0×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
12	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.4×10^3	1.9×10^3	$<1.0 \times 10^1$	2.0×10^1	1.0×10^1	$<1.0 \times 10^1$
13	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.4×10^3	2.3×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
14	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^2	1.7×10^2	1.8×10^2	4.0×10^1	6.0×10^1	$<1.0 \times 10^1$
15	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.4×10^3	2.3×10^3	1.0×10^1	$<1.0 \times 10^1$	4.0×10^1	2.0×10^1

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

[b] Collaborator did not perform direct test method. Data were excluded from statistical analysis of direct test method vs reference method and of direct test method vs resuscitation test method.

Raw Ground Beef

Results are reported in Tables 5A and 5B. Collaborator 6 reported that samples were allowed to thaw either at too high a temperature or for too long a time prior to analysis, resulting in significant overgrowth of *E. coli* O157:H7 by competitive flora in the samples. Collaborator 14 reported growth of *E. coli* O157:H7 in uninoculated control samples by both methods. Data from collaborators 6 and 14 were excluded from statistical analysis. Test method results of collaborators 7 and 12 for the low inoculation level were determined to

Table 6C. Collaborative study results for confirmed *E. coli* O157:H7 counts in frozen whole egg by reference method

	E. coli O157:H7, count/g							
	Lot A[a]		Lot B		Lot C		Lot D	
Lab	1[a]	2	1	2	1	2	1	2
2	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.3×10^2	2.4×10^3	3.6×10^0	$<3.0 \times 10^0$	2.3×10^1	9.1×10^0
4	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.4×10^3	2.4×10^1	3.6×10^0	3.0×10^0	2.3×10^1	9.1×10^0
5	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.6×10^2	2.4×10^3	$<3.0 \times 10^0$	3.6×10^0	$<3.0 \times 10^0$	3.6×10^0
6	$<3.0 \times 10^0$	$<3.0 \times 10^0$	6.0×10^0	9.0×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
7	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.5×10^3	3.5×10^1	9.1×10^0	3.6×10^0	2.3×10^1	2.3×10^1
8[b]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.0×10^0	1.6×10^1	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
9[b]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.1×10^4	4.6×10^3	1.1×10^1	4.3×10^1	4.3×10^1	4.3×10^1
10	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.4×10^3	1.5×10^3	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.6×10^0
12	$<3.0 \times 10^0$	$<3.0 \times 10^0$	4.6×10^3	2.4×10^3	9.3×10^1	9.1×10^0	9.3×10^1	3.9×10^1
13	$<3.0 \times 10^0$	$<3.0 \times 10^0$	4.3×10^2	4.6×10^3	4.3×10^1	$<3.0 \times 10^0$	1.1×10^1	2.3×10^1
14	$<3.0 \times 10^0$	$<3.0 \times 10^0$	5.3×10^2	9.3×10^1	4.3×10^1	3.6×10^0	4.3×10^1	$<3.0 \times 10^0$
15	$<3.0 \times 10^0$	$<3.0 \times 10^0$	4.6×10^3	4.6×10^3	1.5×10^1	9.1×10^0	3.0×10^0	4.3×10^1

[a] Letters refer to the lot from which the sample was drawn: A = uninoculated; B = low level; C = medium level; D = high level. Numbers refer to individual samples from each lot.

[b] Collaborator did not perform direct test method. Data were excluded from statistical analysis of direct test method vs reference method and of direct test method vs resuscitation test method.

Table 7. Confirmation rates of presumptive positive *E. coli* O157:H7 isolates from test and reference methods

Food	Confirmed/tested[a] ratio (%)		
	Direct test method	Resuscitation test method	Reference method
Apple cider	259/267 (97)	N/A[b]	318/364 (87)
Pasteurized milk	255/273 (93)	N/A	344/732 (47)
Cottage cheese	242/255 (95)	N/A	329/415 (79)
Cooked ground pork	164/171 (96)	N/A	224/297 (75)
Raw ground beef	160/171 (94)	N/A	128/203 (63)
Frozen whole egg	129/152 (85)	179/195 (92)	360/459 (78)
Overall	1209/1289 (94)	179/195 (92)	1703/2470 (69)

[a] Number of isolates matching confirming criteria divided by total number of presumptive positive isolates tested. Confirming criteria consisted of lack of yellow pigmentation on TSA and positive agglutination reactions with both O157 and H7 antisera.

[b] N/A, not available. Resuscitation test method was not performed for this food.

be outliers by the Cochran outlier screen and Grubbs extreme value test, respectively, and were excluded from calculations of test method performance. Reference method results from collaborator 4 for the low inoculation level were determined to be outliers by the Cochran outlier screen and were excluded from calculations of reference method performance. The test method produced significantly higher confirmed *E. coli* O157:H7 counts than did the reference method ($Pr > F$ of 0.0263). Repeatability and reproducibility values were consistently lower for the test method than for the reference method at all inoculation levels (*see* Table **997.11A**).

Frozen Whole Egg

Results are reported in Tables 6A–6C. Collaborators 8 and 9 did not perform the direct test method and performed only the resuscitation test method for this product, and their data were excluded from the comparison between direct test method and the reference method but were included in the comparison between the resuscitation test method and the reference method. Results from the direct test method and the resuscitation test method did not differ significantly ($Pr > F$ of 0.1689). Although direct test method results were numerically higher than those of the reference method, the differences were not significant ($Pr > F$ of 0.2131). However, confirmed *E. coli* O157:H7 counts by the resuscitation test method were significantly higher than those obtained by the reference method ($Pr > F$ of 0.0336). Repeatability and reproducibility values for the direct test method were substantially lower than those for the reference method for the low and high inoculation levels and were slightly higher than those for the reference method for the medium inoculation level. Repeatability and reproducibility values for the resuscitation test method were substantially lower than those for the reference method for all inoculation levels (*see* Table **997.11A**).

The test method consists of presumptive enumeration on SD-39 agar followed by confirmation based on O157 and H7 serological tests and on lack of pigment after overnight incubation on TSA. Confirmation rates of presumptive positive colonies from all 6 food products by both test and reference methods are summarized in Table 7. Table 8 shows the percentage of confirmed isolates that also produced typical biochemical results in the completed test phase of the study.

Table 8. Completed test rates of confirmed positive *E. coli* O157:H7 isolates from test and reference methods

Food	Completed/confirmed[a] ratio (%)		
	Direct test method	Resuscitation test method	Reference method
Apple cider	249/259 (96)	N/A[b]	316/318 (99)
Pasteurized milk	247/255 (97)	N/A	351/359 (98)
Cottage cheese	239/242 (99)	N/A	326/329 (99)
Cooked ground pork	158/164 (96)	N/A	216/224 (96)
Raw ground beef	159/160 (99)	N/A	127/128 (99)
Frozen whole egg	129/129 (100)	179/179 (100)	351/360 (98)
Overall	1181/1209 (98)	179/179 (100)	1687/1718 (98)

[a] Number of confirmed positive isolates matching completed test criteria divided by total number of confirmed positive isolates tested. Completed test criteria consisted of an IMViC profile typical of *E. coli*, negative cellobiose fermentation reaction, negative sorbitol fermentation reaction on sorbitol MacConkey agar, and negative fluorescence on HC agar.

[b] N/A, not available. Resuscitation test method was not performed for this food.

Confirmation rates of test method presumptive positive colonies were >90% in all cases, except for frozen whole egg analyzed by the direct test method, which produced a confirmation rate of 85%. The overall test method confirmation rate was 94%. Typically, most or all of the nonconfirming presumptive positive isolates were reported by only one or 2 collaborators for a given food, with the vast majority of collaborators reporting 100% confirmation rates or only a sporadic nonconfirming isolate. Test method completed test rates were ≥96% in all cases and 98% overall.

E. hermanii frequently cross-reacts with O157 and/or H7 antisera. However, it is lysine decarboxylase negative and produces a yellow colony on SD-39 agar. Because of the ability of *E. hermanii* to cross-react serologically, a cellobiose fermentation test was included in the panel of completed tests. The overall completed test rate of 98% indicates that false-positive confirmed test results due to *E. hermanii* were not a significant occurrence. Therefore, the combination of typical appearance on SD-39 agar, typical appearance on TSA, and positive O157 and H7 serological results are sufficient to establish an isolate as *E. coli* O157:H7 without need for additional biochemical tests.

The test method has only 2 critical control points. The incubation temperature is specified as 44.0°–44.5°C and must be closely controlled. Too low an incubation temperature (43°C or less) will permit growth of *H. alvei*, which mimics the appearance of *E. coli* O157:H7 on SD-39 agar. Temperatures above 45.0°C will inhibit growth of *E. coli* O157:H7. The second control point is proper pH adjustment of the SD-39 agar during preparation. This medium is not preadjusted to produce the correct pH automatically, because preadjustment reduces the reliability of the pH-based differential reactions (sorbitol fermentation and lysine decarboxylase). Therefore, careful attention must be paid to instructions for pH adjustment of the medium.

In the precollaborative study prior to the multilaboratory study reported here, each food product was analyzed by both the direct test method and by the resuscitation test method (8). The direct test method produced counts that were either statistically equivalent to or significantly higher than the counts from the reference method. The trend was observed for all food products, except frozen egg, for which the direct test method produced significantly lower counts than did the reference method. Resuscitation test method counts for frozen egg were statistically equivalent to reference method results. Because frozen egg was the only product for which the resuscitation test method was measurably more effective than the direct test method, we decided to confirm this observation by including both the direct test method and the resuscitation test method in the portion of the collaborative study dealing with frozen egg.

The collaborative study results for frozen egg did not confirm precollaborative study observations. Even though the resuscitation test method gave a significantly higher recovery of *E. coli* O157:H7 than the reference method, and even though the direct test method and reference method were statistically equivalent, the direct test method and the resuscitation test method did not differ significantly from each other and numerically were quite similar in their recoveries. Therefore, the direct test method should be adequate for enumeration of *E. coli* O157:H7 in frozen whole egg, eliminating the need for a supplementary 4 h repair step for this food product.

Recommendation

On the basis of this collaborative study, it is recommended that the proposed method for enumerating *E. coli* 0157:H7—based on a hydrophobic grid membrane filter and consisting of 24 h presumptive enumeration on SD-39 agar followed by serological confirmation to obtain a confirmed *E. coli* O157:H7 count—be adopted official first action for meats, poultry, dairy foods, infant formula, liquid eggs, mayonnaise, and apple cider.

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FOOD BIOLOGICAL CONTAMINANTS

LOCATE Enzyme-Linked Immunosorbent Assay for Detection of *Salmonella* in Food: Collaborative Study

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A collaborative study was performed in 27 laboratories to validate the enzyme-linked immunosorbent procedure LOCATE for rapid detection of *Salmonella* in foods. Results were read visually and with a microtiter plate reader. The LOCATE method was compared with the *Bacteriological Analytical Manual* (BAM)/AOAC INTERNATIONAL culture method for detecting *Salmonella* in 6 foods: milk chocolate, nonfat dry milk, dried whole egg, soy flour, ground black pepper, and ground raw turkey. Two foods—dried whole egg and black pepper—required repeat rounds because insufficient data sets were produced initially (AOAC INTERNATIONAL stipulates a minimum of 15 sets per food type). Each laboratory tested one or more of the 6 foods. A total of 1 439 samples were analyzed, and no significant differences ($P < 0.05$) were observed between LOCATE with either visual or reader detection and BAM/AOAC INTERNATIONAL results. The LOCATE screening method with visual or reader detection is recommended for Official First Action Approval.

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The recommendation was approved by the Commitee on Microbiology and Extraneous Materials, and was adopted by the Official Methods Board of the Association. *See* "Official Methods Board Actions" (1997) *J. AOAC Int.* **80**, 126A, and "Official Methods Board Actions" (1997) *Inside Laboratory Management*, November issue.

The official culture method (AOAC Methods **967.28B, 967.26C, 967.27,** and **967.28**) for detecting *Salmonella* in foods is laborious, requiring 4–5 days to obtain negative results (1). The revised method in the *Bacteriological Analytical Manual* (2) requires characterization of atypical colonies in the absence of typical colonies. This method increases the minimum time for negative results to 7–8 days. Enzyme immunosorbent assays (3–5) have been used successfully for rapid screening of foods for *Salmonella*. One such assay is LOCATE, which is convenient and simple to perform because it requires no instrumentation. Wash steps can be performed manually, and assays are read visually or with a microtiter plate reader.

A precollaborative trial with 20 food types indicated that LOCATE is as sensitive as the culture method and that no differences exist visually or instrumentally. On the basis of those results, a collaborative study was performed to compare LOCATE with the conventional culture procedures of AOAC INTERNATIONAL and BAM. The study was designed to meet AOAC INTERNATIONAL requirements for collaborative studies (6).

Collaborative Study

The foods selected for this study were based on previous collaborative studies for rapid *Salmonella* methods (3–5, 7). The foods (nonfat dry milk, dried egg, soy flour, finely ground black pepper, milk chocolate, and raw ground turkey) represent a wide variety of product types incriminated in outbreaks of foodborne

Table 1. *Salmonella* serotypes used

Product	Test organism	Serogroup
Chocolate	*S. senftenberg*	$E_4 : G_{comp}$
Nonfat dry milk	*S. newport*	$C_2 : eh_{comp}$
Black pepper	*S. cubana*	$G_2 : Z_{29}$
Dry egg	*Salmonella* spp.	$B : r : 1_{comp}$
Soy flour	*Salmonella* spp.	$C_2 : Z_{10}en_{comp}$
Turkey	Naturally contaminated	

diseases. Each of the products requires a different preenrichment condition.

Preparation of Salmonella Serotypes

The *Salmonella* serotypes used in the study are listed in Table 1.

Each serotype was propagated separately in 100 mL volumes of trypticase soy broth (TSB) from frozen glycerol stock cultures held at −70°C. TSB broths were incubated at 35°C for 24 h. Cells were collected by centrifugation for 20 min at 15 300 × *g*, washed, and suspended in 10 mL sterile 10% (w/v) reconstituted nonfat dry milk. Cell suspensions were lyophilized in a VirTis Model 10-117 freeze-drying chamber (Gardiner, NY).

Inoculation of Samples

Dry, powdered products (nonfat dry milk, soy flour, black pepper, and dried egg) were inoculated in the same manner. A lyophilized *Salmonella* culture was ground in a mortar and added to a sterile plastic bag containing about 500–1000 g test product. This inoculated ("seeded") product was mixed well by manual agitation and held at room temperature for 2–3 weeks to stabilize the level of *Salmonella* cells. Appropriate portions of seeded product were mixed manually with noninoculated product to obtain target levels of *Salmonella* cells. Milk chocolate seed was prepared by inoculating lyophilized *Salmonella* culture into milk chocolate tempered at 55°C. Sample was mixed with a Kitchen-Aid mixer and held at room temperature for 2–3 weeks to stabilize. Stabilized seed was mixed with tempered chocolate to obtain target levels of *Salmonella* cells.

Raw ground turkey was naturally contaminated. Three batches of turkey were tested and, on the basis of contamination levels, were used as low, medium, and high levels. The turkey was stored at −20°C when initial contamination levels were determined. One day before shipment, samples were thawed and mixed in a Hobart blender for 20 min. Samples of 30–35 g were weighed and frozen at −20°C prior to shipment on dry ice.

Sample Shipment

Collaborators received a set of fifteen 30–35 g samples for each food product. Five samples were inoculated at a high level (10–50 cells/25 g), and 5 at a low level (1–5 cells/25 g). The remaining 5 were uninoculated controls. Samples were shipped via an overnight delivery service. Dry and low-moisture foods were shipped at ambient temperature. Ground turkey samples were shipped frozen. One set of test samples was shipped to each participating laboratory on the Wednesday preceding the Monday that analyses were to be initiated.

Sample Analysis

Each food was scheduled for analysis in a different week. The *Salmonella* level in each food was determined by the most probable number (MPN) procedure using standard culture methods (1). This analysis was initiated in the principal laboratory on the same day the comparative analyses were started. Sample sizes used in triplicate for MPN analysis were 100, 10, 1, and 0.1 g.

Each sample was analyzed by both LOCATE and the BAM/AOAC culture methods. Preenrichment, selective enrichment, isolation, and confirmation of isolates by BAM/AOAC methods were performed as described (1, 2). For all products except ground turkey, the following steps were used for LOCATE. After 22–26 h incubation at 35°C, a separate set of tetrathionate (TT) broth and selenite cystine (SC) broth selective enrichments were inoculated from each preenrichment, i.e., a set independent of that used for the BAM/AOAC method. TT and SC selective enrichments were incubated for 6–8 h at 42° and 35°C, respectively, and 1 mL was transferred from each into separate modified Gram-negative (GN) broths (10 mL/tube). Inoculated GN broths were incubated at 42°C for 18 h. After incubation, 1 mL from each GN broth was combined in a tube and autoclaved at 121°C or boiled for 20 min in a water bath prior to performing the LOCATE assay. TT and SC enrichments were incubated for an additional 16–18 h and used in addition to the GN broths for confirmation of positive assay results. For this study all broth cultures, TT, SC, and both GN broths, were streaked on xylose lysine desoxycholate (XLD), Hektoen enteric (HE), and bismuth sulfite (BS) agars. All subsequent steps in identification and confirmation of presumptive or suspect *Salmonella* isolates were performed by the same methods used for the standard BAM/AOAC culture procedure.

For raw turkey, Rappaport-Vassiliadis (RV) medium replaced SC for both BAM/AOAC and LOCATE methods. For the BAM/AOAC method, RV and TT selective enrichments were incubated at 42° and 43°C, respectively, for 24 h (2). For the LOCATE method, RV and TT were incubated at 42°C for 18–24 h, followed by transfer of 1 mL to separate GN broths. GN broths were then incubated at 42°C for 6 h prior to assay. For culture confirmation, selective enrichments were incubated for an additional 6 h before streaking

Table 997.16A. Statistical analyses of collaborative study results obtained by visual interpretation

Food	Level of *Salmonella* contamination	MPN[b], cfu/g	Total samples	Samples positive: LOCATE Total	LOCATE Suspect	LOCATE Confirmed	BAM/AOAC culture	χ^{2c}	LOCATE performance rates, %: Sensitivity[d]	Incidence of false negatives among total positive samples[e]	Specificity[f]	Incidence of false positives among total negatives samples, %[g]	Agreement[a] between LOCATE and BAM/AOAC methods, %
Milk chocolate	Control[h]	< 0.003	75	0	1	0	0	—[i]	—	—	99	1	100
	Low	0.093	75	72	72	72	72	—	100	0	100	0	100
	High	0.75	75	74	74	74	73	0.50	100	0	100	0	97
Dried whole egg	Control	< 0.003	80	0	1	0	0	—	—	—	99	1	100
	Low	0.043	80	35	35	35	35	—	100	0	100	0	100
	High	0.460	80	79	79	79	79	—	100	0	100	0	100
Nonfat dry milk	Control	< 0.003	74	0	5	0	0	—	—	—	93	7	100
	Low	0.240	75	71	69	68	71	1.33	96	4	99	1	96
	High	0.75	75	75	75	75	75	—	100	0	100	0	100
Black pepper	Control	< 0.003	90	0	3	0	0	—	—	—	97	3	100
	Low	0.004	90	22	24	21	22	0.00	95	5	98	2	99
	High	0.460	90	83	83	83	83	—	100	0	100	0	100
Soy flour	Control	< 0.003	75	0	0	0	0	—	—	—	100	0	100
	Low	0.043	75	42	41	41	41	0.50	98	2	100	0	97
	High	0.240	75	73	73	73	72	0.00	100	0	100	0	99
Raw turkey	Low	< 0.003	85	12	12	10	12	0.00	83	17	97	3	96
	Medium	0.093	85	71	73	71	68	1.33	100	0	98	2	96
	High	2.4	85	80	83	78	79	0.00	98	2	94	6	96

[a] Reflects the number of confirmed determinations that were equivalent for LOCATE and culture methods.

[b] Most probable number of colony-forming units (cfu) per gram of food.

[c] χ^2 is defined by McNemar (9) as $(|a-b|-1)^2/(a+b)$ where a = samples positive by LOCATE and negative by culture method and b = samples negative by LOCATE and positive by culture method. A χ^2 value greater than 3.84 indicates significance at $p < 0.05$.

[d] Sensitivity rate is defined as 100 times the total number of analyzed positive test portions among "known" positive test portions divided by total number of "known" test portions, where "known" positive is defined as samples confirmed positive by the reference method.

[e] Incidence of false negatives is 100 − sensitivity rate.

[f] Specificity rate is defined as 100 times the total number of analyzed negative test portions among "known" negative test portions divided by the total number of "known" negative test portions, where "known" negative is defined as samples confirmed negative by the reference method and negative controls.

[g] Incidence of false positives is 100 − specificity rate.

[h] Uninoculated control samples were by definition known negatives; sensitivity rates were not calculated.

[i] Statistical analysis was not applicable.

Table 997.16B. Statistical analyses of collaborative study results obtained with the automated plate reader

Food	Level of *Salmonella* contamination	MPN[b], cfu/g	Total samples	Samples positive: LOCATE Total	LOCATE Suspect	LOCATE Confirmed	BAM/AOAC culture	χ^{2c}	LOCATE performance rates, %: Sensitivity[d]	Incidence of false negatives among total positive samples[e]	Specificity[f]	Incidence of false positives among total negatives samples, %[g]	Agreement[a] between LOCATE and BAM/AOAC methods, %
Milk chocolate	Control[h]	< 0.003	75	0	1	0	0	—[i]	—	—	99	1	100
	Low	0.093	75	72	72	72	72	—	100	0	100	0	100
	High	0.75	75	74	73	73	73	0.50	99	1	100	0	97
Dried whole egg	Control	< 0.003	80	0	1	0	0	—	—	—	99	1	100
	Low	0.043	80	35	32	32	35	1.33	91	9	100	0	96
	High	0.460	80	79	79	79	79	—	100	0	100	0	100
Nonfat dry milk	Control	< 0.003	74	0	5	0	0	—	—	—	93	7	100
	Low	0.240	75	71	68	67	71	1.33	94	5	99	1	96
	High	0.75	75	75	71	71	75	2.25	95	5	100	0	95
Black pepper	Control	< 0.003	90	0	3	0	0	—	—	—	97	3	100
	Low	0.004	90	22	24	22	22	0.00	100	0	99	1	100
	High	0.460	90	83	83	83	83	—	100	0	100	0	100
Soy flour	Control	< 0.003	75	0	0	0	0	—	—	—	100	0	100
	Low	0.043	75	42	42	41	41	0.50	98	2	99	1	97
	High	0.240	75	73	73	73	72	0.00	100	0	100	0	99
Raw turkey	Low	< 0.003	85	12	12	10	12	0.00	83	17	97	3	96
	Medium	0.093	85	71	73	71	68	1.33	100	0	98	2	96
	High	2.4	85	80	84	78	79	0.00	98	2	92	8	96

[a] Reflects the number of confirmed determinations that were equivalent for LOCATE and culture methods.

[b] Most probable number of colony forming units (cfu) per gram of food.

[c] χ^2 is defined by McNemar (9) as $(|a-b|-1)^2/(a+b)$ where a = samples positive by LOCATE and negative by culture method and b = samples negative by LOCATE and positive by culture method. A χ^2 value greater than 3.84 indicates significance at $p < 0.05$.

[d] Sensitivity rate is defined as 100 times the total number of analyzed positive test portions among "known" positive test portions divided by total number of "known" test portions, where "known" positive is defined as samples confirmed positive by the reference method.

[e] Incidence of false negatives is 100 sensitivity rate.

[f] Specificity rate is defined as 100 times the total number of analyzed negative test portions among "known" negative test portions divided by the total number of "known" negative test portions, where "known" negative is defined as samples confirmed negative by the reference method and negative controls.

[g] Incidence of false positives was 100 specificity rate.

[h] Uninoculated control samples were by definition known negatives; sensitivity rates were not calculated.

[i] Statistical analysis was not applicable.

for isolation and confirmation. All other steps in the analysis of raw turkey were the same as described for other foods.

Analysis of Data

Collaborators were instructed to send all results to the Associate Referee. Data from each food type were collated, numbered, and analyzed for agreement, specificity, sensitivity, false-negative levels, and false-positive levels according to McClure (8).

997.16, *Salmonella* in Foods, Enzyme Immunosorbent Assay

First Action 1997

(Applicable to determination of *Salmonella* in all foods. Because a certain percentage of false-positive and -negative reactions may be encountered, assays must be confirmed by standard culture methods.)

Method Performance:

See Tables **996.16 A** and **B** for method performance data.

A. Principle

Detection of *Salmonella* antigens is based on enzyme immunoassay using specific monoclonal antibodies. Samples to be assayed along with controls are added to wells, and all antigens present will bind onto the well surface. Wells are washed to remove any unbound material. Monoclonal antibody-labeled enzyme (conjugate) is added, and it binds to *Salmonella* antigens if they are present on the surface of the well, thus forming an antibody–antigen complex. Unbound conjugate is removed by washing, and substrate is added. A blue product is formed in the presence of fixed antibody-labeled enzyme conjugate. Results can be read visually, or the reaction can be stopped with acid and the absorbance at 450 nm can be determined with a microplate reader. Samples with values greater than, or equal to, the recommended cut-off value are considered presumptive positive for *Salmonella* antigens.

B. Reagents

Items **(a)**–**(m)** are available as LOCATE *Salmonella* assay kit from Rhône-Diagnostic Technologies Ltd., West of Scotland Science Park, Unit 3.06, Kelvin Campus, Glasgow, G20 OSP Scotland, UK. Store all materials at 2°–8°C. Materials are sufficient for 96 tests.

(a) *96-Well microtiter plate.*—Eight 12-well breakable strips.

Table 2. Test products, test organisms, and inoculation levels

Product	*Salmonella* serovar	Inoculation level	MPN/g
Milk chocolate	—[a]	Control	<0.003
	senftenberg (E_4:G_{comp})	Low	0.093
	senftenberg (E_4:G_{comp})	High	0.75
Dried egg 1	—[a]	Control	<0.003
	B:r:1 complex	Low	0.043
	B:r:1 complex	High	0.43
Dried egg 2	—[a]	Control	<0.003
	B:r:1 complex	Low	0.043
	B:r:1 complex	High	0.460
Nonfat dry milk	—[a]	Control	<0.003
	newport (C_2:eh_{comp})	Low	0.240
	newport (C_2:eh_{comp})	High	0.75
Black pepper 1	—[a]	Control	<0.003
	cubana (G_2:Z_{29})	Low	1.1
	cubana (G_2:Z_{29})	High	11.1
Black pepper 2	—[a]	Control	<0.003
	cubana	Low	0.004
	cubana	High	0.460
Soy flour	—[a]	Control	<0.003
	C_2:Z_{10} en complex	Low	0.043
	C_2:$Z_1$0 en complex	High	0.240
Raw ground turkey[b]	—[c]	Low	<0.003
	C_2:en complex[b]	Medium	0.093
	G:eh:1 complex[b]	High	2.4

[a] —, Uninoculated control.

[b] Naturally contaminated products; predominant serovar listed.

[c] Low level of naturally occurring *Salmonella*.

(b) *Microtiter strip well holder.*—Sufficient for securing individual strips.

(c) *Positive control.*—One vial containing heat-killed, lyophilized *S. poona* (reacts with antibodies to *Salmonella*) with 0.5% L-Bronidox (Henkel KGaA, Germany) as preservative.

(d) *Negative control.*—One vial containing nonreactive heat-killed *Proteus vulgaris* antigens with 0.5% L-Bronidox (Henkel KGaA) as preservative.

(e) *Enzyme conjugate.*—Two vials freeze-dried conjugate containing anti-*Salmonella* antibody conjugated to horse radish peroxidase lyophilized in 0.005 g glycine and 0.028 g sucrose.

(f) *Conjugate diluent.*—One vial (20 mL) Tris-sodium chloride buffer containing 0.01 mL Tween 20 and 1% bovine serum albumin with 0.02% Kathon (Rohm and Haas UK Ltd, Croydon, UK) as preservative.

(g) *Wash buffer concentrate (25×).*—One vial (20 mL) Tris-sodium chloride buffer containing 0.275 mL Tween 20 with 0.2% Kathon (Rohm and Haas UK Ltd) as preservative.

(h) *Substrate.*—One vial (15 mL) TMB (3,3,5,5-tetramethyl benzidine) reagent.

(i) *Stop solution.*—One vial (15 mL) 2% sulfuric acid solution.

(j) *Plate sealer.*

(k) *Modified GN broth (GN broth containing 10 μg/mL novobiocin).*—Ingredients per liter of deionized water: 20 g tryptose, 1 g dextrose, 2 g D-mannitol, 5 g sodium citrate, 0.5 g sodium desoxycholate, 4 g dipotassium phosphate, 1.5 g monopotassium phosphate, 5 g sodium chloride. Prepare novobiocin stock solution (1 mg/mL in water) and filter sterilize. Aseptically add 100 μL novobiocin to each sterile GN broth tube. Novobiocin stock solution may be stored for several weeks in a dark bottle at 4°C.

(l) *Diagnostic reagents.*—Necessary for culture confirmation of assays. *See* AOAC Method **967.27**.

C. Apparatus

(a) *Microplate reader.*—Capable of reading absorbance at 450 nm (optional).

(b) *Incubators.*—35° ± 1°C.

Table 3. Laboratory participation by product type

Laboratory	Milk chocolate	Nonfat dry milk	Dried egg 1	Dried egg 2	Black pepper 1	Black pepper 2	Soy flour	Ground turkey
1	+	+	+	+	+	+	+	+
2	+[a]	+	+[b]	–[c]	+	–[c]	+	+
3	+	+[a]	+[a]	+	+[a]	+	+	+
4	+	+	+	–[c]	+[a]	–[c]	+[a]	+
5	+	+	+	–[c]	+	–[c]	+	+
6	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+[a]	+
8	+	+	+[a]	+	–[c]	–[c]	–[c]	–[c]
9	+[a]	+[a]	+[a]	–[c]	+[a]	–[c]	+	+
10	–[c]	–[c]	+	–[c]	+	+	+	+
11	+	+	+	+	+[a]	+	+	+
12	+	+	+	+	+	+	+	+
13	–[c]	+	+[a]	–[c]	+	–[c]	+	+
14	+	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]
15	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+
18	+	+	+	+	+[a]	+	+	+
19	+	+	+	+	+	+	+	+
20	–[c]	–[c]	–[c]	–[c]	–[c]	+	–[c]	–[c]
21	–[c]	–[c]	–[c]	–[c]	–[c]	+[a]	–[c]	–[c]
22	–[c]	–[c]	–[c]	+	–[c]	+	–[c]	–[c]
23	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	+
24	–[c]	–[c]	–[c]	+	–[c]	+	–[c]	–[c]
25	–[c]	–[c]	–[c]	+[a]	–[c]	+	–[c]	–[c]
26	–[c]	–[c]	–[c]	–[c]	–[c]	+	–[c]	–[c]
27	–[c]	–[c]	–[c]	+	–[c]	–[c]	–[c]	–[c]

[a] Results not used; one or more negative control samples tested positive for *Salmonella* by both LOCATE and culture methods.

[b] Collaborator received samples but did not analyze them.

[c] Laboratory did not receive samples.

(c) *Water baths.*—42° ± 0.5°C.

(d) *Pipettes.*—Capable of delivering 50–100 μL.

(e) *Plastic squeeze bottle to wash wells.*

D. General Instructions

(*1*) Components in kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(*2*) Store all kit components and reagents at 2°–8°C when not in use.

(*3*) Bring reagents to room temperature before use.

(*4*) The substrate TMB (3,3,5,5-tetramethyl benzidine) is light sensitive. Hence, the last stage of color development in the assay should be performed in the dark. TMB should also be stored in the dark at 2°–8°C.

(*5*) Return any remaining strips to the strip holder, cover with the plastic sealer provided, and return to pouch containing dessicant. Fold the pouch and store at 2°–8°C.

Table 4. Collaborative study results for detection of *Salmonella* in chocolate

	Control samples					Low-inoculum samples					High-inoculum samples				
Lab.	2	6	7	12	15	4	5	8	11	14	1	3	9	10	13
	LOCATE visual and reader *Salmonella* assay results, respectively														
1	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
2*	++	++	−−	−−	−−	++	++	++	++	++	++	−+	++	++	++
3	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
4	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
5	+[b] +[b]	−−	−−	−−	−−	++	++	++	++	++	+−	++	++	++	++
6	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
7	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
8	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	+[b] −	++	++
9[a]	++	−−	−−	++	−−	++	++	++	++	++	++	++	++	++	++
11	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
12	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
14	−−	−−	−−	−−	−−	−−	++	++	++	−−	++	++	++	++	++
15	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
16	−−	−−	−−	−−	−−	++	++	++	−−	++	++	++	++	++	++
17	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
18	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
19	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
	LOCATE *Salmonella* confirmation														
1	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
2[a]	++	++	−−	−−	−−	++	++	++	++	++	++	−[c] +	++	++	++
3	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
4	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
5	−−	−−	−−	−−	−−	++	++	++	++	++	+ −[c]	++	++	++	++
6	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
7	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
8	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	−−	++	++
9[a]	++	−[d] −[d]	−−	++	−[d] −[d]	++	++	++	++	++	++	++	++	++	++
11	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
12	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
14	−−	−−	−−	−−	−−	−−	++	++	++	−−	++	++	++	++	++
15	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
16	−−	−−	−−	−−	−−	++	++	++	−−	++	++	++	++	++	++
17	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
18	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
19	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++

Table 4. (*continued*)

	Control samples					Low-inoculum samples					High-inoculum samples				
Lab.	2	6	7	12	15	4	5	8	11	14	1	3	9	10	13
	BAM/AOAC culture method														
1	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
2[a]	–[e]	–[e]	–	–	–	+	+	+	+	+	+	+	+	+	+
3	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
4	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
5	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
6	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
7	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
8	–	–	–	–	–	+	+	+	+	+	+	+	–	+	+
9[a]	–[e]	–	–	–[e]	–	+	+	+	+	+	–[e]	+	+	+	+
11	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
12	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
14	–	–	–	–	–	–	+	+	+	–	+	+	+	+	+
15	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
16	–	–	–	–	–	+	+	+	–	+	+	+	+	+	+
17	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
18	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
19	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+

[a] Results excluded from statistical analysis.
[b] False positive.
[c] LOCATE negative, BAM/AOAC positive, confirmation positive.
[d] LOCATE negative, BAM/AOAC negative, confirmation positive.
[e] False negative.

(*6*) Include one positive and one negative control with each group of test samples.

(*7*) Reconstituted conjugate should be used within 28 days.

(*8*) Different pipette tips must be used for each sample.

(*9*) Incomplete washes will adversely affect test result.

(*10*) Incubation times are crucial. Do not extend or decrease.

(*11*) Treat all materials in contact with bacterial cultures as biohazards and decontaminate appropriately.

E. Sample Preparation

(a) *Preenrichment.*—Preenrich sample in nonselective medium to initiate *Salmonella* growth. Procedure will vary with product type and must be performed as indicated in AOAC Method **967.26** or in *Bacteriological Analytical Manual* (2).

(b) *Selective enrichment.*—Transfer 1 mL incubated preenrichment culture to a tube containing 10 mL SC and 1 mL to a tube containing 10 mL TT broth. For raw or highly contaminated foods, RV medium replaces SC broth, in which case 0.1 mL preenrichment culture is added to 10 mL RV. Incubate according to sample type: (*1*) *Processed foods.*—Incubate SC and TT broths for 6 h at 35° and 42°C, respectively. (*2*) *Raw and highly contaminated products.*—Incubate TT broth and RV medium for 18 ± 2 h at 42°C.

(c) *Postenrichment.*—After selective enrichment incubation, transfer 1 mL TT broth culture to tube containing 10 mL modified GN broth. Transfer 1 mL SC broth culture to separate tube containing 10 mL modified GN broth. Incubate according to sample type: (*1*) *Processed foods.*—Incubate both GN broths for 18 h at 42°C. (*2*) *Raw and highly contaminated products.*—Incubate GN broths for 6 h at 42°C. Reincubate TT broth, SC broth, and RV medium at their respective temperatures for incubation up to a total of 24 ± 2h.

(d) *Preparation of sample for enzyme immunoassay.*—After incubation, transfer 1 mL portions of each modified GN broth into 10 mL test tube, mix combined GN broths, and autoclave or boil for 20 min. Cool broth to room temperature and perform enzyme immunoassay.

F. Assay Procedure

(*1*) Remove number of wells required to perform assay: one well per food sample, one positive control,

and one negative control well. Press these firmly into the tray provided.

(*2*) Add 100 μL controls/well and 100 μL autoclaved or boiled samples/well to the microtiter plate. Record sample positions on the record sheet. Incubate microtiter plates for 30 min at room temperature.

(*3*) Wash each well with a working solution of wash buffer (ca 250 μL per well) and quickly invert plate to empty contents into a container. Repeat this washing process 3 more times with working wash buffer and once with distilled water. Strike the plate face down several times on a paper towel placed on a flat surface.

(*4*) Add 100 μL working conjugate/well to each sample and control well. Incubate for 30 min at 22°–25°C.

(*5*) Wash each well as in step (*3*).

(*6*) Add 100 μL TMB substrate/well. Incubate in the dark at room temperature (22°–25°C) for 30 min.

Table 5. Collaborative study results for detection of *Salmonella* in nonfat dry milk

	Control samples					Low-inoculum samples					High-inoculum samples				
Lab.	4	6	11	12	13	1	3	5	7	9	2	8	10	14	15
	LOCATE visual and reader *Salmonella* assay results, respectively														
1	−−	−−	−−		−−	++	++	++	++	++	++	++	++	++	++
2	−−	−+[a]	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
3[b]	−−	−−	++	−−	−−	++	++	++	++	++	++	++	++	++	++
4	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
5	−−	+[a] +[a]	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++
6	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
7	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
8	−−	−−	−−	−−	+[a] +[a]	++	++	++	++	++	++	++	++	++	++
9[b]	−−	+−	++	++	−−	++	++	++	++	−−	++	++	++	++	++
11	+[a] +[a]	+[a] +[a]	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
12	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
13	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++
15	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
16	+[a] −	−−	−−	−−	−−	−−	−−	+[a] −	−−	+−	+−	+−	+−	++	+−
17	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++
18	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
19	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
	LOCATE *Salmonella* confirmation														
1	−−	−−	−−		−−	++	++	++	++	++	++	++	++	++	++
2	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
3[b]	−−	−−	++	−−	−−	++	++	++	++	++	++	++	++	++	++
4	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
5	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++
6	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
7	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
8	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
9[b]	−[c] −[c]	+−[c]	++	++	−−	++	++	++	++	−−	++	++	++	++	++
11	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
12	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
13	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++
15	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
16	−−	−−	−−	−−	−−	−[d] −[d]	−[d] −[d]	−−	−[d] −[d]	+−[d]	+−[d]	+−[d]	+−[d]	++	+−[d]
17	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++
18	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
19	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++

Table 5. (*continued*)

	Control samples					Low-inoculum samples					High-inoculum samples				
Lab.	4	6	11	12	13	1	3	5	7	9	2	8	10	14	15
						BAM/AOAC culture method									
1	–	–	–		–	+	+	+	+	+	+	+	+	+	+
2	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
3[b]	–	–	+	–	–	+	+	+	+	+	+	+	+	+	+
4	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
5	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
6	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
7	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
8	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
9[b]	–	–[e]	–[e]	–[e]	–	+	+	+	+	–	+	+	+	+	+
11	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
12	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
13	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
15	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
16	–	–	–	–	–	+	+	–	+	+	+	+	+	+	+
17	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
18	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
19	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+

[a] False positive.
[b] Results excluded from statistical analysis.
[c] LOCATE negative, confirmation positive, BAM negative.
[d] LOCATE negative, confirmation positive, BAM positive.
[e] False negative.

(*7*) For visual interpretation, read results immediately after incubation. Do not stop reaction with stopping solution.

(*8*) For automated interpretation, stop reaction with 100 μL stopping solution per well. Read within 5 min. Measure plate absorbance with a microplate reader at 450 nm and record absorbance values on record sheet.

G. Assay Results

(a) *Visual assessment.*—If results are to be determined visually, it must be done prior to addition of stop solution. Positive control should give strong blue color and negative control should be clear or very pale blue. A sample is considered positive when it is bluer in color than the negative control. It is considered negative when its color intensity is equal or less than that of the negative control.

(b) *Automated interpretation.*—Place plate in reader and read at 450 nm. The absorbance at 450 nm (A_{450}) of the negative control should always be < 0.3. Positive control samples must give A_{450} values greater than 1.0. A value greater than 0.3 for negative control indicates insufficient washing. For test sample, $A_{450} \geq 0.3$ indicates a positive sample, and $A_{450} < 0.3$ indicates a negative sample.

For both visual and automated interpretation, negative and positive samples can be interpreted only if the expected results are produced with controls. If controls are not within limits, the test must be repeated.

H. Confirmation

Enrichment broths and GN broths must be streaked on selective media as described in AOAC Method **967.28B**, and typical or suspect colonies must be identified as described in AOAC Methods **967.26C, 967.27,** and **967.28**. As an alternative to conventional tube system (AOAC Method **967.27**) for *Salmonella*, any AOAC-approved commercial biochemical kits may be used for presumptive generic identification of foodborne *Salmonella* as described in AOAC Methods **978.24, 989.12**, and **991.13**.

Ref.: *J. AOAC Int.* **81**, 419 (1998).

Results and Discussion

Salmonella levels in control samples, inoculated samples, and naturally contaminated samples are shown in Table 2. Laboratory participation is shown in Table 3. Each product was sent to 17–19 laboratories. All samples were analyzed. However, some data were ex-

Table 6. Collaborative study results for detection of *Salmonella* in dried egg

	Control samples					Low-inoculum samples					High-inoculum samples				
Lab.	2	5	7	9	10	3	6	8	12	14	1	4	11	13	15
	LOCATE visual and reader *Salmonella* assay results, respectively														
1	−−	−−	−−	−−	−−	++	−−	++	−−	−−	++	++	++	++	++
3	−−	−−	−−	−−	−−	−−	−−	++	+−	+−	++	++	++	++	++
6	−−	−−	−−	−−	−−	++	−−	−−	−−	−−	++	++	++	++	++
7	−−	−−	−−	−−	−−	++	++	−−	−−	−−	++	++	++	++	++
8	−−	−−	−−	−−	−−	++	−−	++	−−	++	++	++	++	++	++
10	−−	−−	−−	−−	−−	++	−−	−−	++	++	++	++	++	++	++
11	−−	−−	−−	+[a] +[a]	−−	−−	++	++	++	−−	++	++	++	++	++
12	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++
15	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++
16	−−	−−	−−	−−	−−	−−	++	++	−−	++	++	++	++	++	++
17	−−	−−	−−	−−	−−	−−	+−	−−	++	+[a] −	++	++	++	++	++
18	−−	−−	−−	−−	−−	++	++	++	++	−−	++	++	++	++	++
19	−−	−−	−−	−−	−−	−−	++	−−	−−	++	++	++	−−	++	++
21	−−	−−	−−	−−	−−	−−	++	++	−−	−−	++	++	++	++	++
22															
23	−−	−−	−−	−−	−−	++	−−	−−	−−	−−	++	++	++	++	++
24	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++
25[b]	++	+[c] +[c]	+[c] +[c]	+[c] +[c]	−−	++	−−	++	−−	++	++	++	++	++	++
	LOCATE *Salmonella* confirmation														
1	−−	−−	−−	−−	−−	++	−−	++	−−	−−	++	++	++	++	++
2															
3	−−	−−	−−	−−	−−	−−	−−	++	+−[d]	+−[d]	++	++	++	++	++
6	−−	−−	−−	−−	−−	++	−−	−−	−−	−−	++	++	++	++	++
7	−−	−−	−−	−−	−−	++	++	−−	−−	−−	++	++	++	++	++
8	−−	−−	−−	−−	−−	++	−−	++	−−	++	++	++	++	++	++
10	−−	−−	−−	−−	−−	++	−−	−−	++	++	++	++	++	++	++
11	−−	−−	−−	−−	−−	−−	++	++	++	−−	++	++	++	++	++
12	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++
15	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++
16	−−	−−	−−	−−	−−	−−	++	++	−−	++	++	++	++	++	++
17	−−	−−	−−	−−	−−	−−	+−[d]	−−	++	−−	++	++	++	++	++
18	−−	−−	−−	−−	−−	++	++	++	++	−−	++	++	++	++	++
19	−−	−−	−−	−−	−−	−−	++	−−	−−	++	++	++	−−	++	++
21	−−	−−	−−	−−	−−	−−	++	++	−−	−−	++	++	++	++	++
23	−−	−−	−−	−−	−−	++	−−	−−	−−	−−	++	++	++	++	++
24	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++
25[b]	++	−−	−−	−−	−−	++	−−	++	−−	++	++	++	++	++	++

Table 6. (*continued*)

	Control samples					Low-inoculum samples					High-inoculum samples				
Lab.	2	5	7	9	10	3	6	8	12	14	1	4	11	13	15
	BAM/AOAC culture method														
1	–	–	–	–	–	+	–	+	–	–	+	+	+	+	+
2															
3	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
6	–	–	–	–	–	+	–	–	–	–	+	+	+	+	+
7	–	–	–	–	–	+	+	–	–	–	+	+	+	+	+
8	–	–	–	–	–	+	–	+	–	+	+	+	+	+	+
10	–	–	–	–	–	+	–	–	+	+	+	+	+	+	+
11	–	–	–	–	–	–	+	+	+	–	+	+	+	+	+
12	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
15	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
16	–	–	–	–	–	–	+	+	–	+	+	+	+	+	+
17	–	–	–	–	–	–	+	–	+	–	+	+	+	+	+
18	–	–	–	–	–	+	+	+	+	–	+	+	+	+	+
19	–	–	–	–	–	–	+	–	–	+	+	+	–	+	+
20															
21	–	–	–	–	–	–	+	+	–	–	+	+	+	+	+
22															
23	–	–	–	–	–	+	–	–	–	–	+	+	+	+	+
24	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
25[b]	+	–	–	–	–	+	–	+	–	+	+	+	+	+	+

[a] False negative.
[b] Results excluded from statistical analysis.
[c] False positive.
[d] LOCATE negative, confirmation positive, BAM positive.

cluded from statistical analyses if one or more negative control samples tested positive for *Salmonella* or if the laboratory deviated from the study instructions. Sample results are shown in Tables 4–9. Method statistics for visual and automated detection assessment are presented in Tables **996.16 A** and **B**, respectively.

Milk Chocolate

Salmonella MPNs determined in milk chocolate samples on the day testing was initiated were 0.093/g and 0.75/g for the low- and high-inoculated samples, respectively (Table 2). Seventeen laboratories analyzed milk chocolate samples, and results from 15 are presented in Table 4. Results from laboratories 2 and 9 were excluded because they show control samples to be positive.

A total of 150 inoculated chocolate samples were tested, 75 at a low level and 75 at a high level. Of these samples, 72 low-inoculum and 73 high-inoculum samples were found to be positive by both LOCATE and BAM/AOAC methods. One high-inoculum sample that was positive by visual assessment but negative by automated assessment was confirmed positive from the broths. Two high-inoculum samples—one a false negative by automated assessment and the other a negative by visual assessment—were both positive by the BAM/AOAC method. One control sample positive by LOCATE both visually and by plate reader and one high-inoculum sample positive visually were not confirmed from the GN broths. The remaining samples were negative by both LOCATE and BAM/AOAC methods. At all test levels, LOCATE visual and automated assessments were not significantly different from results of the BAM/AOAC method ($p < 0.05$, Tables **996.16 A** and **B**).

Nonfat Dry Milk

Salmonella MPNs determined in nonfat dry milk samples were 0.24/g and 0.75/g for the low and high

Table 7. Collaborative study results for detection of *Salmonella* in black pepper

	Control samples					Low-inoculum samples					High-inoculum samples				
Lab.	2	5	8	11	15	1	4	7	10	14	3	6	9	12	13
	LOCATE visual and reader *Salmonella* assay results, respectively														
1	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	−−	++
3	−−	−−	−−	−−	−−	−−	++	−−	++	+[a] −	++	++	++	++	++
6	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	−−
7	−−	−−	−−	−−	−−	−−	++	−−	++	++	++	++	++	++	++
10	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	−−	++
11	+[a] +[a]	+[a] +[a]	−−	+[a] +[a]	−−	−−	−−	−−	−−	+[a] +[a]	++	++	++	++	++
12	−−	−−	−−	−−	−−	++	−−	++	−−	−−	++	++	++	++	++
15	−−	−−	−−	−−	−−	−−	−−	++	++	−−	++	++	++	++	++
16	−−	−−	−−	−−	−−	−−	++	++	−−	−−	−−	++	++	++	++
17	−−	−−	−−	−−	−−	−−	−−	++	−−	−+	++	++	++	++	++
18	−−	−−	−−	−−	−−	++	++	−−	++	−−	++	++	++	++	++
19	−−	−−	−−	−−	−−	++	−−	−−	++	−−	++	++	++	++	++
20	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++
21[b]	−−	−−	−−	++	−−	++	++	++	−−	−−	++	++	++	++	++
22	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	−−	++	++	++
24	−−	−−	−−	−−	−−	−−	−−	++	−−	−−	++	++	++	++	++
25	−−	−−	−−	−−	−−	−−	+[a] +[a]	−−	−−	−−	++	++	++	++	++
26	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++
27	−−	−−	−−	−−	−−	++	−−	−−	−−	−−	++	++	++	++	++
	LOCATE *Salmonella* confirmation														
1	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	−−	++
3	−−	−−	−−	−−	−−	−−	++	−−	++	−−	++	++	++	++	++
6	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	−−
7	−−	−−	−−	−−	−−	−−	++	−−	++	++	++	++	++	++	++
10	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	−−	++
11	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++
12	−−	−−	−−	−−	−−	++	−−	++	−−	−−	++	++	++	++	++
15	−−	−−	−−	−−	−−	−−	−−	++	++	−−	++	++	++	++	++
16	−−	−−	−−	−−	−−	−−	++	++	−−	−−	−−	++	++	++	++
17	−−	−−	−−	−−	−−	−−	−−	++	−−	−[c] +	++	++	++	++	++
18	−−	−−	−−	−−	−−	++	++	−−	++	−−	++	++	++	++	++
19	−−	−−	−−	−−	−−	++	−−	−−	++	−−	++	++	++	++	++
20	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++
21[b]	−−	−−	−−	++	−−	++	++	++	−−	−−	++	++	++	++	++
22	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	−−	++	++	++
24	−−	−−	−−	−−	−−	−−	−−	++	−−	−−	++	++	++	++	++
25	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++
26	−−	−−	−−	−−	−−	−−	−−	−−	−[d] −[d]	−−	−−	−−	++	++	++
27	−−	−−	−−	−−	−−	++	−−	−−	−−	−−	++	++	++	++	++

Table 7. (*continued*)

	Control samples					Low-inoculum samples					High-inoculum samples				
Lab.	2	5	8	11	15	1	4	7	10	14	3	6	9	12	13
	BAM/AOAC culture method														
1	–	–	–	–	–	–	–	–	–	–	+	+	+	–	+
3	–	–	–	–	–	–	+	–	+	–	+	+	+	+	+
6	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–
7	–	–	–	–	–	–	+	–	+	+	+	+	+	+	+
10	–	–	–	–	–	–	–	–	–	–	+	+	+	–	+
11	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
12	–	–	–	–	–	+	–	+	–	–	+	+	+	+	+
15	–	–	–	–	–	–	–	+	+	–	+	+	+	+	+
16	–	–	–	–	–	–	+	+	–	–	–	+	+	+	+
17	–	–	–	–	–	–	–	+	–	+	+	+	+	+	+
18	–	–	–	–	–	+	+	–	+	–	+	+	+	+	+
19	–	–	–	–	–	+	–	–	+	–	+	+	+	+	+
20	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
21[b]	–	–	–	+	–	+	+	+	–	–	+	+	+	+	+
22	–	–	–	–	–	–	–	–	–	+	+	–	+	+	+
24	–	–	–	–	–	–	–	+	–	–	+	+	+	+	+
25	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
26	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
27	–	–	–	–	–	+	–	–	–	–	+	+	+	+	+

[a] False positive.
[b] Results excluded from statistical analysis.
[c] LOCATE negative, confirmation positive, BAM positive.
[d] LOCATE negative, confirmation positive, BAM negative.

levels, respectively (Table 2). Seventeen laboratories analyzed samples (Table 5), of which 15 sent valid data that were used for statistical analysis. Data from laboratories 3 and 9 were excluded because they show uninoculated control samples to be positive. Laboratory 1 had sample 12 missing and did not report results.

A total of 150 inoculated samples were tested, 75 each at low and high inoculum levels. With the exception of results from one laboratory, LOCATE and BAM/AOAC results were identical at both inoculation levels. The exception was laboratory 16, which found 11 samples negative—8 by automated assessment and 3 by visual assessment—all of which were positive by the BAM/AOAC method and were confirmed from assay broths. Of the 75 uninoculated samples, 5 gave false-positive results with LOCATE using visual and automated assessment. One low-inoculum sample was false positive by LOCATE using visual assessment. At all test levels, LOCATE visual and automated assessments were not significantly different from results of the BAM/AOAC methods ($p < 0.05$, Tables **996.16 A** and **B**).

Dried Whole Egg and Black Pepper, Trial 1

Only 13 and 11 valid data sets were obtained initially for dried whole egg and black pepper samples, respectively. AOAC INTERNATIONAL requires at least 15 valid data sets in a qualitative collaborative study, hence trials for both these products were repeated.

Dried Whole Egg, Trial 2

Salmonella MPNs for dried whole egg were 0.043/g and 0.46/g for the low and high levels, respectively (Table 2). Seventeen laboratories analyzed samples

Table 8. Collaborative study results for detection of *Salmonella* in soy flour

	Control samples					Low-inoculum samples					High-inoculum samples				
Lab.	2	5	8	11	14	3	4	9	12	15	1	6	7	10	13
	LOCATE visual and reader *Salmonella* assay results, respectively														
1	−−	−−	−−	−−	−−	++	−−	−−	−−	−+[a]	++	++	++	++	++
2	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
3	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++
4[b]	−−	−−	−−	−−	−−	−−	−−	−−	++	−−	++	++	−−	++	++
5	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
6	−−	−−	−−	−−	−−	−−	++	++	++	−−	++	++	++	++	++
7[b]	−−	−−	−−	−−	++	++	++	++	−−	++	++	++	++	++	++
9	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	++	−−	−−	++	++
10	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++
11	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++
12	−−	−−	−−	−−	−−	++	++	++	−−	++	++	++	++	++	++
13	−−	−−	−−	−−	−−	++	−−	−−	++	−−	++	++	++	++	++
15	−−	−−	−−	−−	−−	−−	++	++	−−	−−	++	++	++	++	++
16	−−	−−	−−	−−	−−	−−	++	++	−−	−−	++	++	++	++	++
17	−−	−−	−−	−−	−−	−−	++	−−	−−	++	++	++	++	++	++
18	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++
19	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
	LOCATE *Salmonella* confirmation														
1	−−	−−	−−	−−	−−	++	−−	−−	−−	−−	++	++	++	++	++
2	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
3	−−	−−	−−	−−	−−	−−	−−	−−	−[c] −[c]	−−	−−	++	++	++	++
4[b]	−−	−−	−−	−[d] −[d]	−−	−−	−−	−−	++	−−	++	++	−−	++	++
5	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
6	−−	−−	−−	−−	−−	−−	++	++	++	−−	++	++	++	++	++
7[b]	−−	−−	−−	−−	++	++	++	++	−−	++	++	++	++	++	++
9	−[e] −[e]	−[e] −[e]	−[e] −[e]	−[e] −[e]	−[e] −[e]	−[e] −[e]	−[e] −[e]	−[e] −[e]	−[e] −[e]	−[e] −[e]	++	−[e] −[e]	−[e] −[e]	++	++
10	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++
11	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++
12	−−	−−	−−	−−	−−	++	++	++	−−	++	++	++	++	++	++
13	−−	−−	−−	−−	−−	++	−−	−−	++	−−	++	++	++	++	++
15	−−	−−	−−	−−	−−	−−	++	++	−−	−−	++	++	++	++	++
16	−−	−−	−−	−−	−−	−−	++	++	−−	−−	++	++	++	++	++
17	−−	−−	−−	−−	−−	−−	++	−−	−−	++	++	++	++	++	++
18	−−	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++
19	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++

(Table 6); results from laboratory 25 were excluded from statistical analysis because they show uninoculated control samples to be positive.

A total of 160 inoculated dried whole egg samples were tested, 80 each at low and high inoculum levels. Three low-inoculum samples gave false-negative results with LOCATE using visual assessment. However, all 3 results were confirmed positive. One control sample gave false-positive results with LOCATE using visual and automated assessments, and one low-inoculum sample gave a false-positive result with LOCATE using visual assessment. For both controls and inoculated samples, LOCATE visual and automated assessments were not significantly different from results of the BAM/AOAC method ($p < 0.05$, Tables **996.16A** and **B**).

Table 8. (*continued*)

	Control samples					Low-inoculum samples					High-inoculum samples				
Lab.	2	5	8	11	14	3	4	9	12	15	1	6	7	10	13
	BAM/AOAC culture method														
1	–	–	–	–	–	+	–	–	–	–	+	+	+	+	+
2	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
3	–	–	–	–	–	–	–	+	–	–	+	+	+	+	+
4[b]	–	–	–	+	–	–	–	–	+	–	+	+	–	+	+
5	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
6	–	–	–	–	–	–	+	+	+	–	+	+	+	+	+
7[b]	–	–	–	–	+	+	+	+	–	+	+	+	+	+	+
9	–	–	–	–	–	–	–	–	–	–	+	–	–	+	+
10	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
11	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
12	–	–	–	–	–	+	+	+	–	+	+	+	+	+	+
13	–	–	–	–	–	+	–	–	+	–	+	+	+	+	+
15	–	–	–	–	–	–	+	+	–	–	+	+	+	+	+
16	–	–	–	–	–	–	+	–[c]	–	–	+	+	+	+	+
17	–	–	–	–	–	–	+	–	–	+	+	+	+	+	+
18	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+
19	–	–	–	–	–	+	+	+	+	+	+	+	+	+	–[c]

[a] False positive.
[b] Results excluded from statistical analysis.
[c] False negative.
[d] LOCATE negative, confirmation positive, BAM positive.
[e] LOCATE negative, confirmation positive, BAM negative.

Black Pepper, Trial 2

Salmonella MPNs for black pepper were 0.004/g and 0.460/g for the low and high levels, respectively (Table 2). Nineteen laboratories analyzed samples (Table 7); results from laboratory 21 were excluded because they show uninoculated controls to be positive.

A total of 180 inoculated samples were tested, 90 each at low and high inoculum levels. LOCATE using visual and automated assessments gave false-results for 3 control samples and 2 low-inoculum samples. Among low-inoculum samples. LOCATE using visual assessment gave in addition, 1 false-positive and 1 false-negative results. One low-inoculum sample found negative by both LOCATE and the BAM/AOAC method was, however, confirmed from GN broths. For this product type, the LOCATE and BAM/AOAC methods were not significantly different ($p < 0.05$, Tables **996.16 A** and **B**).

Soy Flour

Salmonella MPNs determined for soy flour were 0.043/g and 0.240/g for the low and high levels, respectively (Table 2). Seventeen laboratories analyzed samples (Table 8); results from laboratories 4 and 7 were excluded because they show uninoculated controls to be positive.

A total of 150 inoculated soy flour samples were tested, 75 each at low and high inoculum levels. LOCATE gave a false-negative result for one low-inoculum sample. The BAM/AOAC method gave 2 false-negative results, one for a low-inoculum sample and the other for a high-inoculum sample. LOCATE using automated assessment gave a false-positive result for one low-inoculum sample. Laboratory 9 found 12 samples negative by both LOCATE and BAM/AOAC methods. However, the samples were confirmed positive when GN broths were streaked. For this product LOCATE and BAM/AOAC methods were not significantly different ($p < 0.05$, Tables **996.16 A** and **B**).

Ground Turkey

Naturally contaminated turkey meat was used. Results from 17 laboratories are shown in Table 9. Using

Table 9. Collaborative study results for detection of *Salmonella* in turkey

	Low-level contamination					Medium-level contamination					High-level contamination				
Lab.	1	2	6	11	14	4	8	10	12	15	3	5	7	9	13
	LOCATE visual and reader *Salmonella* assay results, respectively														
1	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
2	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
3	−−	−−	−−	++	−−	−−	++	++	++	++	++	++	++	++	++
4	−−	−−	−−	++	−−	++	++	++	++	++	++	++	++	++	++
5	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++	++
6	−−	−−	−−	+[a] +[a]	−−	+[a] +[a]	++	++	++	++	++	++	++	+[a] +[a]	+[a] +[a]
7	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++
9	++	−−	−−	−−	−−	++	++	++	++	++	++	++	++	−+[a]	++
10	−−	−−	−−	−−	−−	++	++	−−	++	++	++	++	++	++	++
11	−−	−−	++	−−	−−	−−	++	−−	++	++	++	++	++	++	++
12	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	−−
13	−−	−−	−−	−−	++	++	−−	−−	++	++	++	++	++	++	++
15	−−	−−	−−	−−	−−	−−	++	−−	++	++	+[a] +[a]	+[a] +[a]	++	+[a] +[a]	++
16	−−	−−	−−	−−	++	++	++	++	−−	++	++	++	++	++	++
17	−−	+[a] +[a]	++	−−	−−	++	++	++	++	++	++	++	++	++	++
18	−−	−−	−−	−−	++	++	++	+[a] +[a]	++	++	++	++	++	++	++
19	−−	−−	++	−−	−−	++	++	++	−−	++	++	++	++	++	++
	LOCATE *Salmonella* confirmation														
1	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
2	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++
3	−−	−−	−−	++	−−	−−	++	++	++	++	++	++	++	++	++
4	−[b] −[b]	−−	−−	++	−−	++	++	++	++	++	++	++	++	++	++
5	−−	−−	−−	−−	++	++	++	++	++	++	++	++	++	++	++
6	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	−−	−−
7	−−	−−	−−	−−	−−	−−	−−	++	++	++	++	++	++	++	++
9	++	−−	−−	−−	−−	++	++	++	++	++	++	++	++	−−	++
10	−−	−−	−−	−−	−−	++	++	−−	++	++	++	++	++	++	++
11	−−	−−	++	−−	−−	−−	++	−−	++	++	++	++	++	++	++
12	−−	−−	−−	−[b] −[b]	−−	++	++	++	++	++	++	++	++	++	−[b] −[b]
13	−−	−−	−−	−−	++	++	−−	−−	++	++	++	++	++	++	++
15	−−	−−	−−	−−	−[c] −[c]	−−	++	−−	++	++	−[c] −[c]	−−	++	−−	++
16	−−	−−	−−	−−	++	++	++	++	−−	++	++	++	++	++	++
17	−−	−−	++	−−	−−	++	++	++	++	++	++	++	++	++	++
18	−−	−−	−−	−−	++	++	++	−−	++	++	++	++	++	++	++
19	−−	−−	++	−−	−−	++	++	++	−−	++	++	++	++	++	++

visual and automated assessment, LOCATE gave 2 false-negative results that were positive by BAM/AOAC and confirmed when GN broths were streaked. Five false-negative results were found by the BAM/AOAC method. The same samples were positive by LOCATE assay and were confirmed when GN broths were streaked. Using visual and automated assessments, LOCATE found 9 false-positive samples. One false-positive result was obtained with automated assessment alone. These differences were not significant ($p < 0.05$, Tables **996.16 A** and **B**).

Recommendation

On the basis of the results of this collaborative study and of the precollaborative study (Curiale and Gangar, unpublished), the LOCATE enzyme immunoassay for detection of *Salmonella* in foods is recommended for Official First Action Approval.

Table 9. (*continued*)

	Low-level contamination					Medium-level contamination					High-level contamination				
Lab.	1	2	6	11	14	4	8	10	12	15	3	5	7	9	13
	BAM/AOAC culture method														
1	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
2	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
3	–	–	–	+	–	–	+	+	+	+	+	+	+	+	+
4	+	–	–	+	–	+	+	+	+	+	+	+	+	+	+
5	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+
6	–	–	–	–	–	–	+	+	+	+	+	+	+	–	–
7	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
9	–[c]	–	–	–	–	+	+	+	+	+	+	+	+	–	+
10	–	–	–	–	–	+	+	–	+	+	+	+	+	+	+
11	–	–	+	–	–	–	+	–	+	+	+	+	+	+	+
12	–	–	–	+	–	+	+	+	+	+	+	+	+	+	+
13	–	–	–	–	+	–[c]	–	–	+	+	+	+	+	+	+
15	–	–	–	–	+	–	+	–	+	+	+	–	–[c]	–	+
16	–	–	–	–	+	+	+	+	–	+	+	+	+	+	+
17	–	–	+	–	–	+	+	+	+	+	+	+	+	+	+
18	–	–	–	–	+	+	–[c]	–	–[c]	+	+	+	+	+	+
19	–	–	+	–	–	+	+	+	–	+	+	+	+	+	+

[a] False positive.
[b] LOCATE negative, confirmation positive, BAM positive.
[c] False negative.

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FOOD BIOLOGICAL CONTAMINANTS

Comparison of the VITEK® Gram-Negative Identification Card and an Enhanced Version for Identification of *Salmonella, Escherichia coli*, and Other *Enterobacteriaceae*: Method Modification

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The VITEK® Gram-Negative Identification Card (GNI) was compared to an enhanced version of the test kit, the GNI+ Card. The GNI Card is an official AOAC method (991.13) for identification of *Salmonella, Escherichia coli*, and other *Enterobacteriaceae* in foods. In this comparison 5 replicates of 124 gram-negative stock culture strains were evaluated to determine equivalency. Isolates were obtained primarily from food sources, with 108 of the isolates representing 15 genera within the family *Enterobacteriaceae*. Overall agreement between the GNI Card and the GNI+ Card was 98.2%. Of the 23 strains of *Salmonella* tested, the longest identification time for the GNI Card was 10 h, with 43.5% of the strains identifying in 4 h or less. The longest identification time of the same *Salmonella* strains tested with the GNI+ Card was 8 h, with 95.7% of the strains identifying within 4 h. The GNI+ Card provided increased speed of identification while retaining the accuracy of the GNI Card. The GNI method for identifying *Salmonella, Escherichia coli*, and other *Enterobacteriaceae* was approved as a method modification by AOAC INTERNATIONAL.

The objective of this study was to compare the performance of the VITEK Gram-Negative Identification Card (GNI) to an enhanced version (GNI+). The GNI Card is AOAC Official Method **991.13**, *Salmonella, Escherichia coli*, and Other *Enterobacteriaceae* in Foods (1). It is a test procedure for presumptive generic identification of foodborne *Salmonella* and for screening and elimination of non-*Salmonella* isolates. It is a suitable alternative to conventional biochemical tests for identifying *E. coli* from foods and may be used for presumptive generic identification of other *Enterobacteriaceae* from foods. The method received First Action in 1991 and Final Action in 1994.

The manufacturer of the GNI card, bioMérieux Vitek, Inc., has redeveloped the GNI Card to improve performance and increase speed of identification. The formulations of the 30 existing biochemical substrates in the GNI Card were optimized in the improved GNI+ Card.

The base medium composition was optimized to limit indicator–substrate–enzymatic interactions that generated nonreproducible reactive patterns. Formulation improvements were made by using several strategies: (*1*) balancing the ratio of existing indicator and buffer components of each reaction for enhanced sensitivity in the VITEK System, (*2*) refining the substrate and cofactor concentrations required for enhanced speed and robustness of performance, and (*3*) optimizing base medium composition to promote reactivity of preformed enzyme systems.

A data base of 3179 strains from 167 species was used in refining software analysis rules. Hourly performance reviewed against these optimized, faster reactions resulted in more rapid identifications. Software analysis of the reformulated GNI+ Card results in identification of glucose-fermenting organisms in 2 to 8 h and of non-glucose fermenting organisms in 4 to 12 h.

In this equivalency study, bioMérieux Vitek, Inc., undertook an internal evaluation modeled after the design of the original collaborative study (2).

Materials and Methods

Five replicates of 124 gram-negative pure stock cultures were tested on the GNI Card and the GNI+ Card. The test organisms were primarily of food origin (Table 1). Thirty-two American Type Culture Collection (ATCC) strains and 7 clinical stock strains were included in the evaluation. The study consisted of 23

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The recommendation was approved by the Commitee on Microbiology and Extraneous Materials, and was adopted by the Official Methods Board of the Association. *See* "Official Methods Board Actions" (1997) *J. AOAC Int.* **80**, 126A, and "Official Methods Board Actions" (1997) *Inside Laboratory Management*, November issue.

Table 1. Source of bacterial strains

Organism	No. of strains	Source
Salmonella spp.	23	Beef, poultry, milk, pork, cheese, flour, whey, ice cream, ATCC
Escherichia coli	13	Beef, pork, milk, soup, buckwheat flour, O157:H7, ATCC
Citrobacter spp.	7	Pork, beef, tomato products, alfalfa, ATCC
Edwardsiella spp.	3	ATCC, clinical
Enterobacter spp.	13	Beef, pork, soup, tomato products, trail mix, mushrooms, batter, flour, ATCC
Pantoea agglomerans	4	Parsley, peaches, sunflower seeds, ATCC
Shigella spp.	8	ATCC, clinical
Hafnia spp.	3	Beef, ATCC
Klebsiella spp.	8	Beef, milk, pudding, cornmeal, ATCC
Proteus spp.	6	Tomato products, beef, poultry, ATCC
Providencia spp.	7	Soup, ATCC, clinical
Yersinia spp.	7	Shell fish, ATCC, clinical
Serratia spp.	4	Creamer, soup, beets, ATCC
Morganella morganii	1	ATCC
Leclercia adecarboxylata	1	ATCC
Non-*Enterobacteriaceae*	16	ATCC, clinical

Salmonella strains representing 21 species, 13 *E. coli* strains, 72 other *Enterobacteriaceae* strains, and 16 non-*Enterobacteriaceae* isolates (Table 2). The test kits were inoculated according to manufacturer's instructions. In all inoculations, a GNI Card and a GNI+ Card were paired for filling from the same bacterial saline suspension, and cards were tested in parallel in the automated VITEK System.

Most organisms tested were the same stock strains used in the original collaborative study. They were stored at −70°C in tryptic soy broth containing 15% glycerol. To demonstrate that storage conditions had not altered biochemical expression, conventional biochemical tube testing was conducted on a subset of strains selected at random.

Results

Overall agreement between the GNI Card and the GNI+ Card was 98.2%. The GNI identified 98.9% of the tested strains correctly; the GNI+ identified 99.0% of the tested strains correctly. Of the 23 strains (21 species) of *Salmonella* tested (115 GNI Cards and 115 GNI+ Cards), the longest identification time for the GNI was 10 h, with 43.5% of the strains identified in 4 h or less. The longest identification time of the same *Salmonella* strains for the GNI+ was 8 h, with 95.7% of the strains identified within 4 h. The GNI gave 115 correct *Salmonella* identifications; the GNI+ gave 112 correct *Salmonella* identifications and 3 unidentified results. No *Salmonella* strains were misidentified by either test kit (Table 2).

Of 13 *E. coli* strains tested (65 GNI Cards and 65 GNI+ Cards), the longest identification time for the GNI was 13 h, with 61.5% of the strains identified in 4 h or less. The longest identification time of the same *E. coli* strains for the GNI+ was 8 h, with 84.6% of the strains identified within 4 h. Both Cards gave 65 correct identifications (Table 2).

Of 72 strains of other *Enterobacteriaceae* tested, representing 13 genera and 30 species (360 GNI Cards and 360 GNI+ Cards), the longest identification time for the GNI was 13 h, with 21.9% of the strains identified within 4 h. The longest identified time of the same strains for the GNI+ was 8 h, with 65.0% of the strains identified within 4 h. The GNI produced 356 correct identifications, one misidentification for *Shigella sonnei*, 2 misidentifications for *Enterobacter cloacae*, and one unidentified result for *Yersinia kristensenii*. The GNI+ yielded 357 correct identifications and 3 misidentifications: one for *Yersinia kristensenii* and 2 for *Yersinia frederickseni* (Table 2).

Of 16 strains of gram-negative non-*Enterobacteriaceae* tested (80 GNI Cards and 80 GNI+ Cards), the GNI produced 77 correct identifications and 3 unidentified results. The GNI+ yielded 80 correct identifications (Table 2).

A subset of 14 random strains tested in conventional tubes produced expected reactions for all 27 biochemicals tested (3).

Summary

The performance of an improved test kit, the Vitek GNI+ Card, was compared to AOAC Official Method **991.13**, *Salmonella*, *Escherichia coli*, and Other *Enterobacteriaceae* in Foods, Biochemical System Identification (VITEK GNI). Five replicates of 124 stock culture strains primarily of food origin were tested, representing a total of 620 GNI Cards and 620 GNI+ Cards. The GNI Card identified 98.9% of the tested strains correctly; the GNI+ Card identified 99.0% of the tested strains correctly. Overall agreement between the 2 cards was 98.2%. According to Fisher's exact test to compare performance, the accuracies of the 2 test kits were not significantly different ($p = 0.999$). Given the overall agreement rate of 98.2%, it can be said with 95% confidence that the true agreement rate between the 2 test kits for the sampled population is no less than 97.1%. On the basis of these results, the GNI+

Table 2. Summary of results

Organism	No. of strains	GNI			GNI+			
		Correct	Misidentification	No identification	Correct	Misidentification	No identification	Total
Salmonella spp.	23	115	0	0	112	0	3	115
Escherichia coli	13	65	0	0	65	0	0	65
Other *Enterobacteriaceae*								
Citrobacter freundii	4	20	0	0	20	0	0	20
Citrobacter koseri	2	10	0	0	10	0	0	10
Citrobacter amalonaticus	1	5	0	0	5	0	0	5
Proteus mirabilis	4	20	0	0	20	0	0	20
Proteus vulgaris	2	10	0	0	10	0	0	10
Klebsiella pneumoniae	6	30	0	0	30	0	0	30
Klebsiella oxytoca	2	10	0	0	10	0	0	10
Shigella sonnei	4	19	1	0	20	0	0	20
Shigella dysenteriae	2	10	0	0	10	0	0	10
Shigella boydii	1	5	0	0	5	0	0	5
Shigella flexneri	1	5	0	0	5	0	0	5
Edwardsiella tarda	3	15	0	0	15	0	0	15
Yersinia kristensenii	3	14	0	1	14	1	0	15
Yersinia enterocolitica	2	10	0	0	10	0	0	10
Yersinia fredericksenii	1	5	0	0	3	2	0	5
Yersinia intermedia	1	5	0	0	5	0	0	5
Enterobacter cloacae	8	38	2	0	40	0	0	40
Enterobacter sakazakii	3	15	0	0	15	0	0	15
Enterobacter aerogenes	1	5	0	0	5	0	0	5
Enterobacter amnigenus bio 1	1	5	0	0	5	0	0	5
Pantoea agglomerans	4	20	0	0	20	0	0	20
Providencia stuartii	3	15	0	0	15	0	0	15
Providencia alcalifaciens	2	10	0	0	10	0	0	10
Providencia rettgeri	2	10	0	0	10	0	0	10
Serratia liquefaciens	1	5	0	0	5	0	0	5
Serratia marcescens	2	10	0	0	10	0	0	10
Serratia odorifera	1	5	0	0	5	0	0	5
Morganella morganii	1	5	0	0	5	0	0	5
Leclercia adecarboxylata	1	5	0	0	5	0	0	5
Hafnia alvei	3	15	0	0	15	0	0	15
Non-*Enterobacteriaceae*	16	77	0	3	80	0	0	80
Total No. of strains	124	613	3	4	614	3	3	620

kit gives shorter identification times than does the GNI while retaining the accuracy of the GNI (Method **991.13**).

Storage conditions of the strains used in the original collaborative study and retested in this evaluation did not alter biochemical expression of the organisms, as determined by conventional tube biochemical testing.

Recommendation

On the basis of the results of this study, we recommend that the GNI method for identifying *Salmonella*, *Escherichia coli*, and other *Enterobacteriaceae* be adopted as a minor method modification to AOAC Official Method **991.13**.

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FOOD CHEMICAL CONTAMINANTS

Determination of Diarrheic Shellfish Toxins in Mussels by Microliquid Chromatography–Tandem Mass Spectrometry[1]

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A fast, sensitive, and specific procedure for determining toxins that cause diarrheic shellfish poisoning (DSP) using microliquid chromatography coupled with tandem mass spectrometry (micro-LC-MS-MS) is reported. The lipophylic polyether acidic toxins okadaic acid (OA), its isomer dinophysistoxin-2 (DTX-2), the 35-methyl-okadaic acid dinophysistoxin-1 (DTX-1), and the novel toxin dinophysistoxin-2B (DTX-2B; recently isolated from Irish mussels) were extracted from shellfish tissues with acetone and chromatographed by isocratic elution at 10 μL/min with CH_3CN–H_2O, 80 + 20 (v/v), containing 0.1% trifluoroacetic acid, through a C_{18} reversed-phase column (1.0 mm id). The chromatograph is coupled via an ion spray interface to an atmospheric pressure ionization source. Collision-induced-dissociation (CID) ion mass spectra of the protonated molecule, $[M + H]^+$, at *m/z* 805 for OA, DTX-2, and DTX-2B and at *m/z* 819 for DTX-1, were obtained in MS-MS experiments to identify 2 diagnostic fragment ions for each analyte that could be used for selected-reaction-monitoring (SRM) micro-LC-MS-MS analysis. The CID spectrum of DTX-2B confirmed it to be a new OA isomer, like DTX-2. Standard curves obtained by SRM micro-LC-MS-MS were linear ($r^2 \geq 0.9992$) over the range 0.05–1.00 μg/mL (i.e., 0.10–2.00 μg toxin/g hepatopancreas), and a detection limit of 15 pg/injection was obtained for each DSP toxin. Average recoveries ranged from 95 to 101%, and coefficients of variation ranged from 1.8 to 3.4%. This novel SRM micro-LC-MS-MS method was used to confirm acidic DSP toxins in Irish and Italian toxic mussels. It offers a high degree of specificity because analyte confirmation is based on retention time, molecular weight, structural information obtained from the presence of 2 diagnostic fragments for each analyte, and ion ratios. OA was found in both Irish (≤0.7 μg/g hepatopancreas) and Italian (≤1.5 μg/g hepatopancreas) mussels. DTX-1 was found only in Italian mussels (≤0.3 μg/g hepatopancreas). DTX-2 (≤6.1 μg/g hepatopancreas) and DTX-2B (≤0.08 μg/g hepatopancreas) were unique to Irish shellfish.

Compounds that cause diarrheic shellfish poisoning (DSP) are a class of chemically and toxicologically different dinoflagellate toxins in the aquatic food chain. They pose serious problems both to human health and to the shellfish industry, because of the marked increase in many parts of the world, particularly in Japan and in Europe, of diseases resulting from human ingestion of contaminated shellfish. Three groups of lipophylic polyether compounds have been isolated from shellfish and dinoflagellates and their structures elucidated: acidic toxins, including the polyether carboxylic acid okadaic acid (OA; 1–3) and its analogues, the dinophysistoxins (DTXs; 4–7), pectenotoxins (PTXs; 5, 6, 8); and yessotoxin (YTX) and its analogues (8–10). Although YTX has been found in Norwegian shellfish (11) and PTX-2 recently has been identified in phytoplankton in Italy (12), OA, is responsible for most outbreaks of DSP in Europe (13, 14).

The 35-methylokadaic acid dinophysistoxin-1 (DTX-1) is found rarely in Europe (15), with the exception of a report of high levels of DTX-1 in Norwegian shellfish (16). The main acute toxic effect of OA and DTX-1 is gastroenteritis, but they also are potent tumor promoters (17). These physiological effects are probably due to their potent inhibitory effect on protein phosphatase (18), but the precise mechanisms of action remain to be elucidated (19).

Other acidic toxins have been discovered. The OA isomer dinophysistoxin-2 (DTX-2; 20) has been identified in mussels from Ireland, where it continues to be the predominant toxin (21, 22). It is also found in mussels from Spain (23) and Portugal (24). The 7-O-acylated toxins (DTX-3) have been found in European

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shellfish (25–27). Recently, we isolated a novel toxin belonging to the OA group, dinophysistoxin-2B (DTX-2B), from Irish mussels (28).

The complexity and variety of DSP toxins in European seafood require a sensitive and specific analytical procedure to confirm identities of toxins and to identify new toxins. Biological, chemical, and immunoassay methods have been developed to detect DSP toxins in mussels, the most common being the mouse bioassay (29). However, this bioassay is a nonselective method with low sensitivity. Although it is widely used for routine monitoring of shellfish for toxin contamination, it provides little information about toxin composition.

Chemical methods to detect DSP toxins, detection such as liquid chromatography (LC) with fluorescence detection of DSP derivatives or LC coupled with mass spectrometry (LC-MS), are valuable in characterizing toxin profiles of phytoplankton and shellfish. Although LC with fluorescence detection (30, 31) has advantages in speed, accuracy, and sensitivity, its use is hampered by the lack of pure analytical standards for DSP toxins. At present, LC-MS is the most powerful technique for confirming identities of known toxins and identifying new compounds (12, 15, 31–36). LC-MS is also becoming a popular technique with the appearance of relatively inexpensive instruments.

The aim of this work was to determine toxic DSP compounds present in mussels from Italy and Ireland by microliquid chromatography–tandem mass spectrometry (micro-LC-MS-MS). Microcolumns facilitate determination of samples with extremely small volumes and/or low concentrations of analytes (37, 38) and are particularly useful because some toxins, such as DTX-2 and DTX-2B, are not commercially available and must be isolated from limited contaminated shellfish samples. The method has a high degree of specificity and sensitivity for OA, DTX-1, DTX-2, and the new toxin DTX-2B.

Experimental

Materials

(a) *Solvents.*—LC reagent grade acetonitrile, methanol, acetone, and trifluoroacetic acid (TFA) (Farmitalia Carlo Erba, Milan, Italy). Water was purified in a Milli-Q system (Millipore Corp., Bedford, MA).

(b) *DSP toxins.*—OA (>95% purity) and DTX-1 (>98% purity) were purchased (Calbiochem-Novabiochem, San Diego, CA). DTX-2 and DTX-2B were isolated from naturally contaminated Irish mussels (≤1.6 μg DTX-2/g hepatopancreas, ≤0.08 μg DTX-2B/g hepatopancreas) as described elsewhere (28, 39). DTX-2 and DTX-2B were >95% pure on the basis of LC after derivatization with 1-bromoacetylpyrene (40). The high degrees of purity were confirmed by flow injection analysis (FIA) MS (scanning from m/z 400 to m/z 1700) of DTX-2 and DTX-2B solutions in positive-ion mode.

Safety notes: LD_{50} <500 mg/kg is known for OA and DTX-1. These compounds may be carcinogenic and/or teratogenic.

(c) *Standard solutions.*—Individual standard stock solutions containing DTX-2, DTX-2B, and DTX-1 at 5 μg/mL were obtained by dilution of pure toxins with methanol. Calibration solutions containing 0.05, 0.10, 0.25, 0.50, and 1.00 μg/mL and standard solutions for spiking, containing each toxin at 0.10 and 0.50 μg/mL, were prepared by dilution of individual standard stock solutions with methanol.

(d) *Mobile phase.*—Acetonitrile–water (80 + 20) containing 0.1% TFA at a flow rate of 10 μL/min.

Apparatus

(a) *LC system.*—A high-pressure pump, model Phoenix 20 CU (Fisons, Milan, Italy), a Valco valve (Valco, Houston, TX) with 1 μL internal loop and a microcolumn (30 cm × 1 mm id stainless steel) packed with 5 μm Supelcosil LC_{18}-DB (Supelco, Bellefonte, PA) were used.

(b) *Mass spectrometer.*—PE-SCIEX API III plus triple quadrupole (PE-Sciex, Thornhill, Ontario, Canada) equipped with an atmospheric pressure ionization (API) source and an ion spray interface set at 5500 V. Ultra-high-purity nitrogen was used as curtain gas and nebulizer gas in the ion spray interface. Orifice potential voltage (OR) was set at 50 V. MS and MS-MS experiments were run with a resolution of 0.8 (measured at half height) for both resolving quadrupoles. In MS-MS experiments, collision-induced-dissociation (CID) spectra were acquired by colliding quadrupole 1 (Q1) selected precursor ion with argon gas in Q2, operated in radiofrequency RF-only mode, and scanning the second quadrupole mass spectrometer, Q3. Collision energy of 20 eV was used for CID experiments. The data system with standard software packages—Tune version 2.1.2, Macspec version 3.2, Macquan version 1.1.2 (PE-Sciex)—was used for instrument control, data acquisition, data manipulation, and peak area integration. The protonated molecule, $[M + H]^+$, of each toxin was the precursor ion for CID, and diagnostic daughter ions for each analyte were identified for selected-reaction-monitoring (SRM) micro-LC-MS-MS analysis. This was implemented with parent–daughter ion combinations of m/z 805–751 and m/z 805–769 for OA, DTX-2, and DTX-2B and m/z 819–765 and m/z 819–784 for DTX-1. In all cases in this paper, m/z values are the truncated values of the more accurate experimental values.

Sample Preparation

(a) *Blank control samples.*—Mussels used for recovery studies were collected from batches available on

the market and analyzed by micro-LC-MS-MS to verify absence of toxins before spiking. The digestive glands (hepatopancreas) were removed and stored at −20°C until used.

(b) *Toxic samples.*—Irish toxic mussels were collected in 1994 from coastal farms in southwest Ireland, following toxin detection (22) in routine screening using fluorimetric LC (30). Italian mussels were collected from mussel farms of northern coastal areas of the Adriatic sea in 1994, when routine control tests with Yasumoto's biological assay (29) had shown mussels to be positive for DSP toxins. Hepatopancreas were removed and stored at −20°C prior to extraction.

(c) *Spiked control samples for recovery studies.*—Homogenized hepatopancreas (1.0 g) from blank control samples was mixed with standard toxin solutions to obtain spiked control samples containing 0.2 and 1.0 μg of each toxin/g hepatopancreas. Recovery was determined by SRM micro-LC-MS-MS. The amount of each toxin extracted from mussels was determined by comparison with the standard curve.

(d) *Extraction of toxins from mussel hepatopancreas.*—Homogenized hepatopancreas (1.0 g) was extracted 3 times at room temperature with 8.00 mL acetone for 2 min. Combined acetone extract was filtered and evaporated to dryness on a rotary evaporator with a temperature-controlled bath (40°C). Residue was suspended in 2.00 mL methanol and centrifuged at $1000 \times g$ for 10 min. Then, 1 μL was injected into the LC-MS instrument.

Because of the low level of DTX-2B, extract from ca 10 g mussel hepatopancreas was taken up in ether and applied to a silica column (10 g, silica gel 60, 70–230 mesh; E. Merck, Darmstadt, Germany). The column was washed with ether (10 mL), the wash was discarded, and then the DSP toxins were eluted with methanol–ether (5 + 95; 15 mL) to yield a fraction containing OA, DTX-2, and DTX-2B. Further elution with methanol–ether (15 + 85; 30 mL) gave only OA and DTX-2.

Results and Discussion

Ion spray MS is a powerful technique for determining DSP toxins (12, 15, 26, 31–33, 36). Full-scan mass spectra acquired in single MS positive-ion mode, showed the protonated molecules $[M + H]^+$ at m/z 805 for OA, DTX-2, and DTX-2B and at m/z 819 for DTX-1, with no evidence of fragmentation (28).

The observation of molecular mass information is one of the most important criteria for identification, but diagnostic ions also are required. In this work, the intact protonated molecule of each analyte served as the precursor ion for CID in MS-MS experiments, which were performed first by FIA on individual toxin standards.

Figure 1 shows positive-daughter mass spectra (range m/z 10–850) of protonated OA, DTX-2, DTX-2B, and DTX-1. As expected, OA and DTX-1 spectra confirmed the fragmentation previously obtained by Quilliam (31) through LC-MS-MS experiments with an API source and an ion spray interface. The spectrum of the OA isomer, DTX-2, gave the same fragment ions as OA. The similarity between the OA and DTX-2 fragment ion mass spectra can be interpreted by considering the molecular structures of the two isomeric toxins and the previously reported OA fragmentation pattern (31, 34, 35). The latter shows the fragment ion at m/z 169, arisen from cleavage of the C-29–C-30 bond, to contain the rings in which the methyl group, at C-31 for OA and C-35 for DTX-2, is located. The CID spectrum of DTX-2B, obtained for the first time, gave the same fragmentation pattern as OA and DTX-2, thus showing that DTX-2B is an isomer of OA and DTX-2. Data from repeated analyses under different collision energies in both positive- and negative-ion fragmentation modes, support the conclusion of structural similarities among DTX-2B, OA, and DTX-2.

These MS-MS spectra provided very useful structural information and fragment ions for confirmatory analysis in SRM mode, which was implemented by using the parent–daughter ion combinations of m/z 805–751 and m/z 805–769 for OA, DTX-2, and DTX-2B and m/z 819–765 and m/z 819–784 for DTX-1. In agreement with previous researchers (15, 31), we improved the signal-to-noise ratio for all acidic toxins under investigation after decreasing the flow

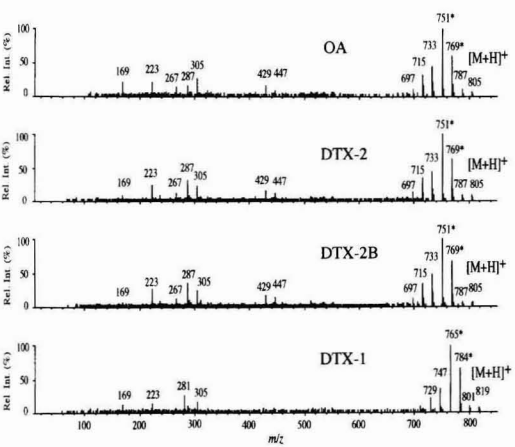

Figure 1. Positive product ion mass spectra of protonated OA (m/z 805), DTX-2 (m/z 805), DTX-2B (m/z 805), and DTX-1 (m/z 819). CID was performed with a collision energy of 20 eV and argon as collision gas. Analyses by FIA MS-MS with ions monitored for confirmation by selected-reaction-monitoring (SRM) LC-MS-MS are indicated with asterisks.

rate of mobile phase during FIA-MS-MS experiments; the best sensitivity was achieved at 10 μL/min. However, our results show that FIA-MS-MS is not selective enough to differentiate OA, DTX-2, and DTX-2B, because no significant differences were observed among the relative intensities of the same fragment ions in the CID spectra of the 3 toxins.

Chromatographic separation of analytes therefore was required. We used a micro-LC_{18} column (1 mm id) with an optimum binary mobile phase of aqueous acetonitrile with 0.1% TFA, which permitted low-flow LC-MS-MS without column eluate splitting.

Under these conditions, SRM micro-LC-MS-MS analyses of a mixture of OA, DTX-2, DTX-2B, and DTX-1 (0.5 μg/mL of each toxin) gave excellent separation, the toxins eluting at 26.3, 28.8, 31.3, and 34.1 min, respectively (Figure 2A). Good chromatographic separation among the 4 analytes was obtained with the same mobile phase at higher flow rates (20–50 μL/min). Thus, analysis time can be reduced by a factor of 4. However, under such conditions, sensitivity decreased considerably. Therefore, flow rate of 10 μL/min was selected for maximum sensitivity.

For all analytes, a detection limit of 15 pg injected (i.e., 15 ng/mL) was obtained, on the basis of a signal-to-noise ratio of at least 3:1 on any single ion chosen for SRM analysis (Figure 2B). This improvement in instrumental detection limit for determination of OA analogues corresponds to a method detection limit of about 30 ng/g hepatopancreas, when the simplified method for extraction is applied. Further concentration of extracts after cleanup can lower detection limits even more (31). Sample enrichment allows a further 10–15-fold improvement of the detection limit.

An internal standard is generally useful in order to obtain precise LC-MS-MS quantitative data. Although 7-*O*-acetylokadaic acid has been proposed recently (31) as an internal standard for determination of OA in mussels by ion spray LC-MS, the compound is not available commercially. We have found that external calibration can be suitable for quantitative analysis of DSP toxins by ion spray LC-MS-MS, provided that the vacuum chamber pressure is controlled accurately to avoid instrument drift.

Data from standard calibration curves, covering the range 0.05–1.00 μg/mL (i.e., 0.10–2.00 μg toxin/g hepatopancreas) for the 4 toxins are shown in Table 1. Good correlation coefficients ($r^2 \geq 0.9992$) indicate excellent linearity. Recovery and precision data were generated each day for 3 days from analysis of duplicate control mussel hepatopancreas samples spiked with each toxin at 0.2 and 1.0 μg/g. SRM micro-LC-MS-MS

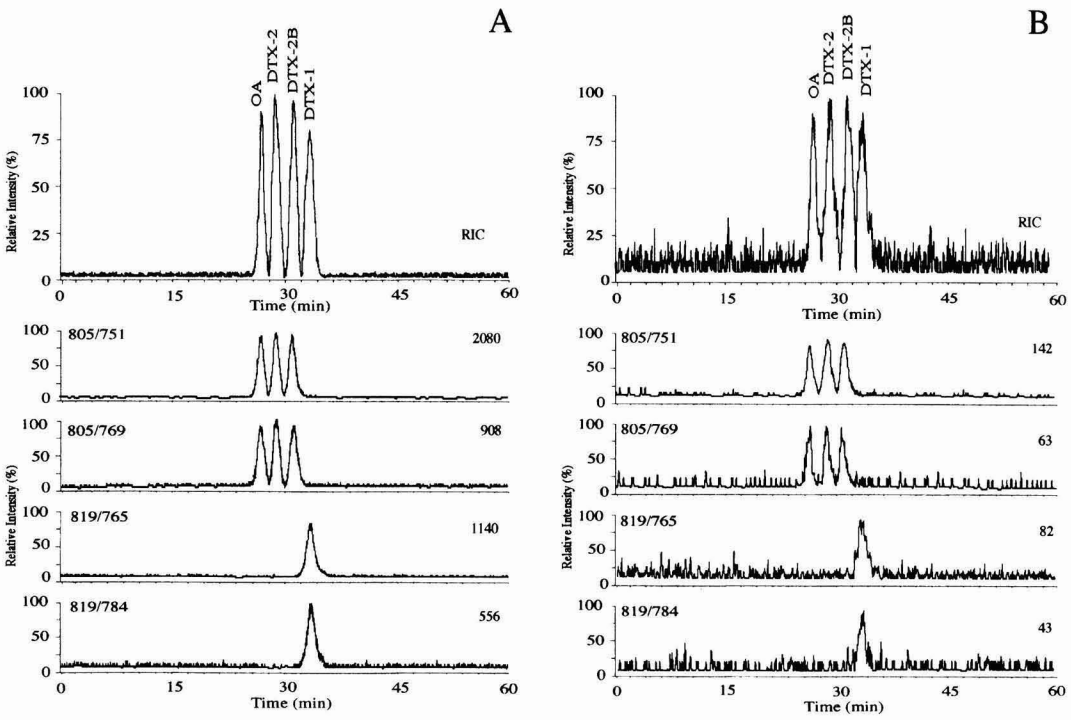

Figure 2. SRM LC-MS-MS chromatograms of a mixture of OA, DTX-2, DTX-2B, and DTX-1. (A) 500 pg of each toxin was injected. (B) Limit of detection (15 pg of each toxin injected). Intensity counts are shown at the upper right of each chromatogram.

Table 1. Correlation coefficient (r^2), intercept, and slope of linearity plots used for quantitation of toxins

Toxin	r^2	Intercept	Slope
OA	0.9999	−132.4	28483
DTX-2	0.9999	−52.1	29312
DTX-2B	0.9996	203.5	30306
DTX-1	0.9992	−76.7	21250

chromatograms of blank and spiked control samples of mussel hepatopancreas showed no interferences around analyte retention times. Results of recovery studies are presented in Table 2. Recoveries (95.4–101.1%) were satisfactory at each level for all toxins. Precision data also were excellent for all toxins at all levels, with coefficients of variation ranging from 1.8 to 3.4%. Retention times of all toxin chromatographic peaks in spiked control samples were within 1% of reference standards, and diagnostic ion ratios for each toxin were within the 13% of corresponding ratios for the DSP toxin standard.

Micro-LC-MS-MS analysis of extracts from hepatopancreas of toxic Italian and Irish mussels confirmed the presence of OA ($\leq$1.5 μg/g hepatopancreas) and DTX-1 ($\leq$0.3 μg/g hepatopancreas) in Italian mussels (Figure 3A). In Irish mussels, OA ($\leq$0.7 μg/g hepatopancreas), DTX-2 ($\leq$6.1 μg/g hepatopancreas), and DTX-2B ($\leq$0.08 μg/g hepatopancreas) were detected but not DTX-1. Figure 3B shows the chromatogram obtained for determination of DTX-2B in an Irish shellfish extract after concentration and cleanup using silica. Accuracy and precision values in Table 2 refer only to simplifed extraction procedure, and these data were compiled from mussel samples spiked with toxins.

The SRM micro-LC-MS-MS method has a high degree of specificity, because analyte confirmation is based on retention time, molecular weight, and structural information, such as presence of 2 diagnostic fragments for each analyte and ion ratios. Interestingly, the same confirmatory ions obtained by CID in the collision cell during MS-MS experiments also were produced by CID between the ion sampling orifice and the mass analysis region, the so called up-front CID, by increasing the OR to 70 V. The single-quadrupole instrument therefore could be a less expensive alternative to MS-MS for routine confirmation of OA, DTX-2,

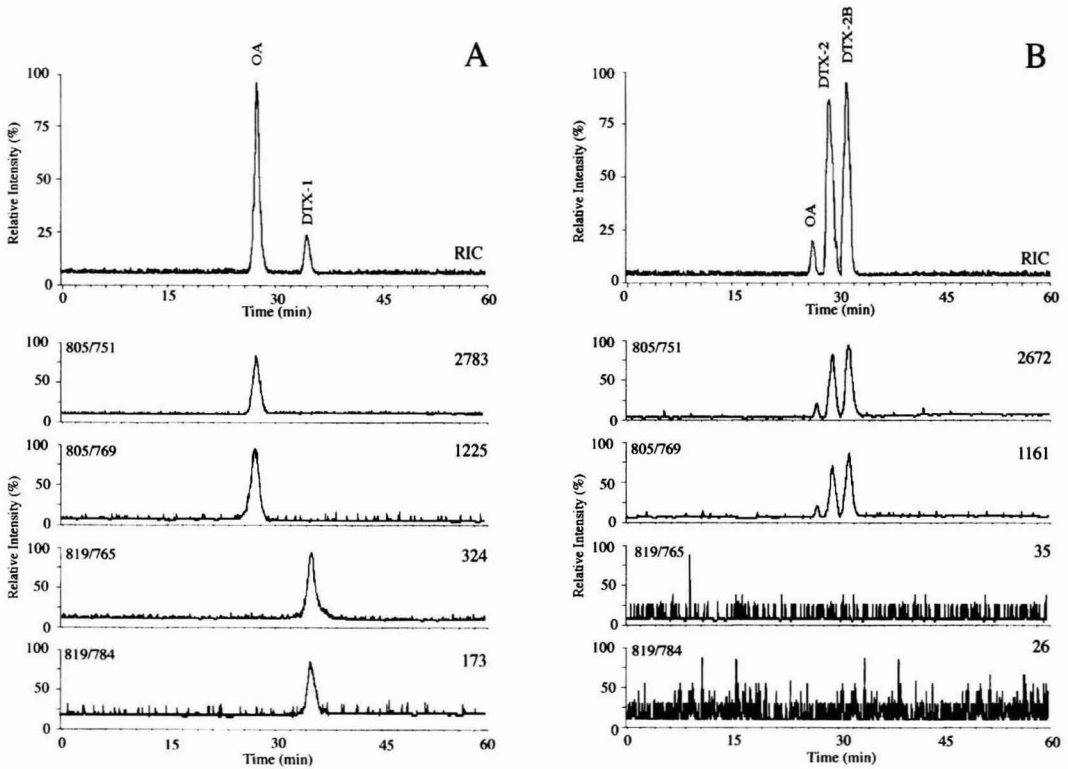

Figure 3. SRM LC-MS-MS chromatograms of extracts from hepatopancreas of Italian and Irish toxic mussels. (A) Italian mussel: OA (1.51 μg/g), DTX-1 (0.29 μg/g). (B) Irish mussel extract fraction after cleanup on silica: DTX-2B (0.080 μg/g). Intensity counts are shown at the upper right of each chromatogram.

Table 2. Recovery of toxins from spiked mussel hepatopancreas

Toxin	Level of spike, μg/g	Recovery, %[a]	CV, %
OA	0.2	100.3	3.2
	1.0	101.1	1.8
DTX-2	0.2	98.6	2.4
	1.0	99.8	2.1
DTX-2B	0.2	97.7	3.1
	1.0	97.8	2.4
DTX-1	0.2	95.4	3.3
	1.0	99.0	3.4

[a] Each value is the average of 6 samples (2/day for 3 days).

DTX-2B, and DTX-1, according to previous reports (31).

The role of OA and DTX-1 in tumor promotion (17) and the low availability of some DSP toxin standards mean that sensitive analytical methods are required for identifying and determining these analytes in small samples. The SRM micro-LC-MS-MS method, which has a lower detection limit compared with the ion spray LC-MS method, should be a valuable analytical tool.

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FOOD CHEMICAL CONTAMINANTS

One-Step Solid-Phase Extraction Cleanup and Fluorometric Analysis of Deoxynivalenol in Grains

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A rapid, quantitative, inexpensive, efficient method was developed to determine deoxynivalenol (DON) in wheat, barley, corn, wheat middlings, wheat flour, bran, malted barley, and oats. Samples are ground and extracted with acetonitrile–water (86 + 14). A portion of the extract is cleaned up by passage through a MycoSep No. 225 column, evaporated to dryness, and derivatized with zirconyl nitrate and ethylenediamine in methanol. The resulting fluorescent derivative of DON is identified and quantitated with a calibrated fluorometer containing a broad wavelength pulsed xenon light source. This method quantitated DON concentrations from 0.5 to 50 ppm without dilution and was linear when applied to samples of noncontaminated wheat spiked at 0.5, 5, 10, 25, and 50 μg DON/g. Correlation coefficients of the method with LC for multiple analyses ($n \geq 14$ for each commodity) applied to wheat, corn, barley, wheat flour, and wheat middlings were 0.99, 0.99, 0.99, 0.93, and 0.98, respectively. Individual analyses were conducted in < 30 min, and 24 samples were analyzed in 2 h.

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8 one; DON) is a member of the sesquiterpenoid group of fungal metabolites known as trichothecenes and is characterized by a ketone moiety at the C8 position. This feature distinguishes Group B from Group A trichothecenes. DON is also known as vomitoxin because of its association with vomiting and feed refusal in swine fed grains contaminated with the producing fungus *Fusarium graminearum* (teleomorph = *Giberella zeae)* (1, 2). Although this organism is the most common producer (3), *F. culmorum* also produces this toxic metabolite. Deoxynivalenol is stable, survives processing (milling), and can occur in foods prepared from contaminated grains (4). DON can occur in grains in association with its precursors/metabolites, 15-acetyldeoxynivalenol and 3-acetyldeoxynivalenol.

Recently in the United States, there has been considerable contamination of wheat with deoxynivalenol because of the occurrence of *Fusarium* head blight (scab) in crops in various areas of the country (5). The fungus *F. graminearum* is the agent of the disease, and recent studies in wheat have demonstrated that DON is associated with the disease-producing potential of this organism (6, 7). This widespread occurrence of the disease in these crops and the associated contamination of grain with DON has caused increased awareness and a need for testing grains for this mycotoxin. Current methods of analysis of DON in cereal grain and foods include thin-layer chromatography (TLC), gas chromatography (GC), liquid chromatography (LC), and enzyme-linked immunosorbent assay (ELISA). The TLC method of Trucksess et al. (8) was collaboratively studied and is an official First Action method of AOAC INTERNATIONAL. This method lacks the sensitivity of other methods but is very useful as a semiquantitative or screening method for DON. Bennett and coworkers (9) described a more sensitive GC procedure with electron capture detection of the heptafluorobutyryl derivative of DON. This method was modified and collaboratively studied (10) and is an official AOAC method. However, this method is rather labor-intensive and not suitable for field testing. Also, an LC procedure (11) is quite effective for analysis of DON in grains with UV detection particularly with efficient cleanup using a MycoSep No. 225 column (Romer Labs, Inc., Union, MO) (12). This cleanup procedure is included in the method of Trucksess et al. (13). However, although this method is sensitive, it is not suitable for field investigations and takes considerable procedural time.

The newest technology for DON assays is the use of monoclonal antibodies for an ELISA to detect DON in grain samples and cereal grain food products (14). Although this is a fairly simple test to perform there can be some cross reactivity of the antibody to similar compounds, and dilution of sample extracts are required if the concentration of DON is > 5 ppm. Also, to limit the expense of the test and to shorten the time of assay, samples must be grouped before an analysis can be economically efficient.

This report describes the use of a one-step solid-phase extraction cleanup column followed by a unique fluorometric method for determination of DON in

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grains. This quantitative method is rapid, accurate (similar to LC) from 0.5 to 50 ppm with no dilutions, economical for individual samples, inexpensive, and approved by the U.S. Department of Agriculture, Grain Inspections, Packers and Stockyards Administration.

METHOD

Apparatus

(a) *Fluorometer.*—Model FX-100 (Romer Labs, Inc., Union, MO) equipped with broad wavelength pulsed Xenon lamp, selected source filters (365–380 nm excitation, 450–550 nm emission), silicon detector, and RS-232C output for printer.

(b) *Liquid chromatograph.*—Shimadzu LC-10A with an Sil-10A autoinjector, SPD-10A UV-Vis detector at 220 nm, SCL-10A system controller, and RP-18 column (100 × 3 mm) fitted with RP-18 guard column (15 × 3 mm).

(c) *Grinding mill.*—Romer grinding/subsampling mill, Series II (Romer Labs, Inc.).

(d) *Blender.*—Osterizer 14 speed blender (Sunbeam-Oster Household Products, Schaumburg, IL).

(e) *Blender jars.*—One-half pint, glass jars suitable for use with blender.

(f) *Vortex mixer.*—Model G-560 (Scientific Industries, Inc., Bohemia, NY).

(g) *Evaporator.*—Romer Evap (Romer Labs, Inc.).

(h) *Dry baths.*—Two dry bath incubators with 12-place heating blocks for 15 × 85 mm culture tubes.

(i) *Repipettor.*—Eppendorf Repeator Pipette 4780.

(j) *Pipettor.*—Finnpipette, 200–1000 mL.

(k) *Filter paper.*—Whatman No. 4 filter paper.

(l) *Glass culture tubes.*—15 × 85 mm borosilicate, nonsterile (Kimble Glass, Vineland, NJ).

(m) *Glass cuvettes.*—12 × 75 mm borosilicate fitted with Uni-Flex safety caps (12 × 13 mm, Bio Plas, Inc., San Francisco, CA).

Reagents

(a) *Solvents.*—Acetonitrile for extraction, reagent grade (Mallinkrodt Chemical Works, St. Louis, MO). Acetonitrile and methanol for LC and all other solutions, LC grade (Mallinkrodt Chemical Works).

(b) *Reagent A.*—Ethylenediamine in methanol; 0.04% (Romer Labs, Inc.).

(c) *Reagent B.*—Zirconyl nitrate in methanol; 3.75% (Romer Labs, Inc.).

(d) *DON standards.*—DON in methanol (Romer Labs, Inc.).

(e) *Calibrators.*—*(1) Low calibrator.*—DON (derivatized with reagents A and B) in acetonitrile at concentration equivalent to background fluorescence obtained from wheat sample containing no detectable DON by LC analysis. *(2) High calibrator.*—Same as low calibrator, except DON concentration is equivalent to that of noncontaminated wheat sample spiked at 5 μg/g.

(f) *Control solution.*—Known concentration of DON in acetonitrile–water, 86 + 14 (Romer Labs, Inc.).

(g) *Cleanup columns.*—MycoSep No. 225 columns (Romer Labs, Inc.).

Sample Preparation and Extraction

Prior to extraction, representative samples of grain (3 lb wheat or similar sized grain or 5 lb corn) were ground with the Romer mill. Samples of wheat middlings, bran, flour, and similar finely ground materials can be analyzed without further grinding. A 25 g subsample from the front chute on the mill, or other materials as noted above, was placed in a blender jar along with 100 mL (150 mL for wheat middlings and bran) acetonitrile–water (86 + 14). The extraction/blending was conducted for 3 min at high speed in the blender. The extraction solvent was decanted into a glass container through filter paper.

Cleanup

A 4 mL portion of the extract was placed in a 15 × 85 mm test tube; the MycoSep column was inserted into the top of the test tube and slowly pushed (30 s time elapse, except 40 s was used for wheat middlings and bran) to the bottom of the tube.

Derivatization

The solution above the column packing was mixed with repeated filling and discharging from the pipettor, and 1.5 mL of the purified sample extract was then transferred to a cuvette. After this sample was evaporated to complete dryness in a dry bath at 65°C on the evaporator, it was derivatized by adding 1.5 mL reagent A and 50 μL reagent B to yield a fluorescent compound according to the method of Kato et al. (15). The tube was placed on a Vortex shaker for 10 s and heated for 10 min at 50°C in a dry bath. The cuvette was cooled to room temperature by placing in cool tap water for 30 s, and was then placed in the fluorometer.

Fluorometry

The fluorometer was calibrated before use with the high and low calibrators. Two calibrators (high and low) were prepared for this test. Similar calibrators were originally prepared for all commodities, but subsequently only the wheat-based calibrators were used, and the calibration factors on the fluorometer were adjusted to compensate for these matrix differences. After the fluorometer was calibrated, the sample was read using direct readout in decimals and ppm. The

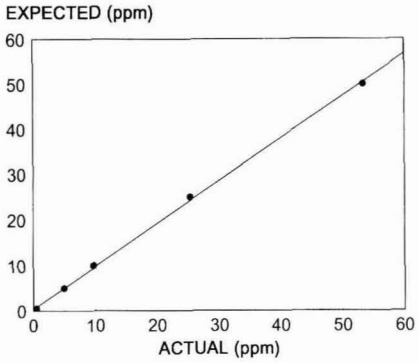

Figure 1. Linearity of fluorometric measurement of derivatized DON in wheat spiked at 0.5, 5, 10, 25, and 50 μg/g.

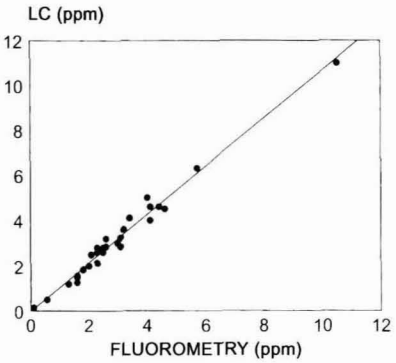

Figure 2. Fluorometric measurement of DON compared to LC analysis in 29 naturally contaminated wheat samples. Correlation coefficient = 0.99.

control solution was analyzed along with the test samples. Commodities and products that have been analyzed using this fluorometric procedure include wheat, wheat flour, bran, middlings and germ, corn, barley, malted barley, and oats. Comparisons with LC analysis were conducted on wheat, wheat flour, wheat middlings, corn, and barley.

LC Analysis

The LC procedure was that of Trucksess et al. (13). For this procedure, 2 mL filtered extract was evaporated to dryness; the residue was dissolved in 0.5 mL mobile phase (water–acetonitrile–methanol, 90 + 5 + 5), mixed thoroughly, and 100 mL was injected. The flow rate was 1 mL/min. The limit of detection of the detector at 220 nm was 0.2 mg; the sensitivity of the method was 100 ng/g.

Results and Discussion

To examine the procedure for linearity, 5 samples of wheat containing no detectable DON were spiked at 0.5, 5, 10, 25, and 50 μg DON/g, and tested. The results were plotted versus the actual values of the spike (Figure 1). All spiked samples tested very close to the actual values. The linearity from 0.5 to 50 ppm using this method is sufficient to determine any significant levels of naturally occurring DON in grains and their products.

The results of testing 29 samples of naturally contaminated wheat by this fluorometric procedure were very close to those of LC (correlation coefficient = 0.99) applied to the same samples (Figure 2). Similar correlation coefficients were obtained when naturally contaminated samples (15 corn, 14 barley, 21 wheat middlings, and 25 wheat flour) were analyzed by the same 2 procedures (Figures 3–6).

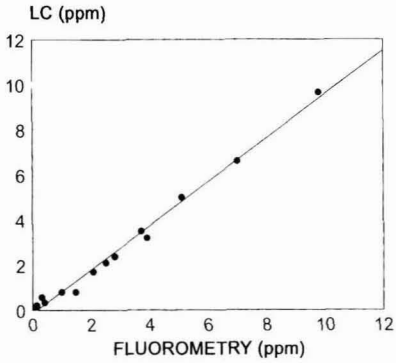

Figure 3. Fluorometric measurement of DON compared to LC analysis in 15 naturally contaminated corn samples. Correlation coefficient = 0.99.

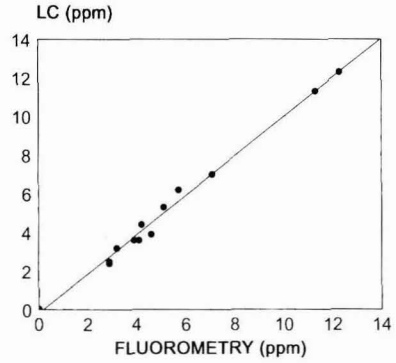

Figure 4. Fluorometric measurement of DON compared to HPLC analysis in 14 naturally contaminated barley samples. Correlation coefficient = 0.99.

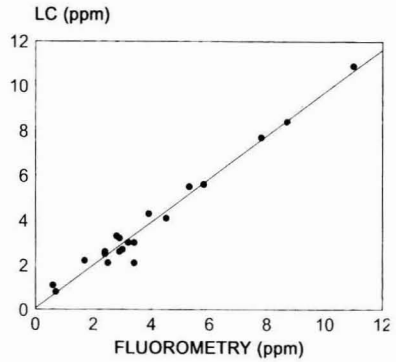

Figure 5. Fluorometric measurement of DON compared to HPLC analysis in 21 naturally contaminated wheat middlings samples. Correlation coefficient = 0.98.

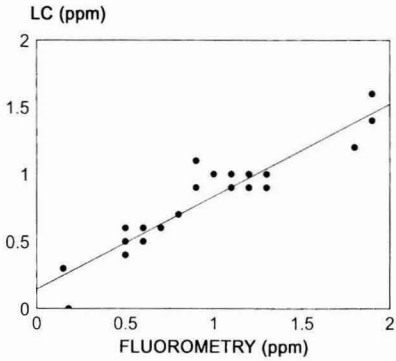

Figure 6. Fluorometric measurement of DON compared to HPLC analysis in 25 naturally contaminated wheat flour samples. Correlation coefficient = 0.93.

The entire procedure for a single sample took <30 min to conduct, and 24 samples were analyzed in 2 h. Using an ELISA method, Xu et al. (16) completed only a single test in <2 h, although current ELISA methods are much more rapid. However, this technique is not as adaptable for single sample analysis as the described fluorometric procedure. Samples would have to be grouped to make the ELISA test more efficient both economically and timewise. Because the fluorometric procedure is rapid and grouping of samples is not required, it can be applied to individual truckloads of grain arriving at buying stations or terminals. Thus, delays for unloading trucks at terminals caused by the time for analysis can be decreased significantly.

The extraction efficiency of DON from molded rice using different solvents was studied by Tanaka et al. (17), and acetonitrile–water (3 + 1) recovered the greatest amount of DON. However, we found that by increasing the amount of acetonitrile in the extraction solvent there was less extraction of interfering substances and similar extraction efficiency of DON from naturally contaminated samples (data not shown). Thus, we used acetonitrile–water (86 + 14) in this procedure. Use of the one-half pint jar for extraction/blending was required to get adequate mixing of the entire sample during the extraction. Larger vessels allowed sample materials to splash and cling to areas of the vessel not washed by the solvent and therefore not extracted entirely.

The sample cleanup procedure using the MycoSep No. 225 column was sufficient for derivatization and analysis of DON in the wide range of commodities and products tested with this fluorometric procedure. Because of the constituency of the columns no shelf life and refrigeration were necessary.

Specificity was very similar to LC analysis for DON, and thus the fluorescent derivatization must be considerably specific for DON. That is, at least the major fluorescent is due to the DON derivative and not to the closely related precursors/metabolites such as 3-acetyl and 15-acetyldeoxynivalenol. Kato et al. (14) reported that the reagents would react with fusarenon-X and nivalenol, making them fluorescent.

The results of this study clearly demonstrate that this fluorometric method is an efficient, accurate, and rapid procedure for the analysis of DON in grains and their products.

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FOOD COMPOSITION AND ADDITIVES

Zero Control Reference Materials for Infant Formula Methods Development

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A zero control reference material (ZRM) for milk and soy-based infant formula was manufactured and characterized. The ZRM was free of retinyl palmitate and all-rac-α-tocopheryl acetate. The composition was similar to commercially available infant formula. The ZRM provides a valuable tool to ascertain method performance.

Protocols for analytical method development use standard addition as an intrinsic aspect of the development process. Essentially, the analyte exists in the matrix to which an identical known pure standard is added. The spiked and non-spiked matrix are extracted and analyzed. By spiking at increasing levels, the researcher establishes, to some degree of certainty, the recovery and response linearity of the standard additions. Data are also evaluated to determine reproducibility, precision, accuracy, and other quality assurance parameters. Unfortunately, standard addition does not allow evaluation of the method at analyte concentrations lower than the endogenous level (1).

Nutrient analysis methods developed by the standard addition technique lead to a dilemma from a regulatory perspective. For example, some nutrients have an upper as well as a lower legal value. Nutrient claims at >100% of label claim can be confidently evaluated by such methods. Nutrient values <100% of label claim complicate regulatory decisions because questions cannot be answered about performance at levels <100% of label claim.

To solve this dilemma, a concept known as Zero Control Reference Material (ZRM) was recently introduced (1). ZRM is a product matrix that lacks those fortified nutrients that are to be assayed. The use of ZRM in method development enables an investigator to validate the method at nutrient levels approaching zero. This report details the manufacture and characterization of ZRM for milk and soy-based infant formula useful in methods development for analysis of retinyl palmitate and all-rac-α-tocopheryl acetate. This study used a previously developed method (2) to characterize the ZRM for retinyl palmitate and all-rac-α-tocopheryl acetate.

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METHOD

Apparatus

(a) *Mixer.*—Univex mixer Model M12B, Food Process Research and Development Laboratory, Department of Food Science and Technology, University of Georgia (UGA), Athens, GA.

(b) *Homogenizer.*—Gavlin, Model 15 MR-87A (UGA), with one pass at 6000 psi.

(c) *Pasteurizer.*—Cherry-Burrell, Model 40 (UGA), at 225°C with a 5 s hold.

(d) *Spray drier.*—A/S Niro Atomizer (UGA), with inlet temperature 275°C and outlet temperature 115°C.

(e) *Liquid chromatograph.*—LDC Analytical Constametric 3200 pump (Thermo Separation Products, Riviera Beach, FL) and a Waters 715 autoinjector (Waters, Inc., Milford, MA).

(f) *Column.*—Lichrosorb Si 60, 5 μm, 4.6 mm × 25 cm (E. Merck, Darmstadt, Germany).

(g) *Integrator.*—Model 3396 or equivalent (Hewlett-Packard, Atlanta, GA). A computerized data system is best.

(h) *Fluorescence detector.*—Model 1046A programmable fluorescence detector (Hewlett-Packard) or equivalent.

(i) *Polytron homogenizer.*—Kinematica PT10-35 (Brinkman Instruments, Westbury, NY).

(j) *Rotary evaporator.*—Buchi Rotavapor EL 130 (Brinkman Instruments).

(k) *Turboevaporator.*—Turbo Vap II (Zymark, Hopkinton, MA).

(l) *Nylon filter.*—0.45 μm (Universal Scientific, Inc., Atlanta, GA).

Reagents

(a) *Hexane.*—LC grade (Burdick & Jackson, Muskegon, MI). Dry the hexane over molecular sieves before use.

(b) *Isopropanol.*—LC grade (EM Science, Gibbstown, NJ).

(c) *Ethyl acetate.*—LC grade (Burdick & Jackson).

(d) *Magnesium sulfate.*—Anhydrous (Fisher Chemical, Fairlawn, NJ).

(e) *Mobile phase.*—Isopropanol at 0.5% in hexane for all-rac-α-tocopheryl acetate analysis and isopropanol at 0.125% in hexane for retinyl palmitate analysis.

(f) *Butylated hydroxytoluene (BHT).*—Weigh ca 9 mg BHT (Sigma Chemical Co., St. Louis, MO) into 25.0 mL volumetric flask and dilute to volume with hexane for concentration of ca 360 μg/mL.

(g) *Retinyl palmitate stock standard solution.*—Accurately weigh ca 50 mg retinyl palmitate (Fluka Bio Chemika, Switzerland) into 50.0 mL volumetric flask and dilute to volume with hexane. Determine exact concentration from $E^{1\%}_{1cm}$ value of 975. Make appropriate dilutions with respective mobile phase to give 5 working standard concentrations ranging from 0.30 to 6.0 μg/mL.

(h) *All-rac-α-tocopheryl acetate stock standard solution.*—Accurately weigh ca 200 mg all-rac-α-tocopheryl acetate (Fluka Bio Chemika) into 50.0 mL volumetric flask and dilute to volume with hexane. Determine exact concentration from $E^{1\%}_{1cm}$ value of 42. Make appropriate dilutions with respective mobile phase to give 5 working standard concentrations ranging from 2.0 to 60 μg/mL.

(i) *Extraction solution.*—Hexane–ethyl acetate, 85 + 15, v/v.

(j) *Zero control ingredients.*—(*1*) *Lactose.*—Monohydrate crystalline (Sigma Chemical Co.); (*2*) *Carrageenan.*—Type II (Sigma Chemical Co.); (*3*) *Ascorbic acid.*—Sodium salt (Sigma Chemical Co.); (*4*) *Taurine.*—Sigma Chemical Co.; (*5*) *Coconut oil.*—Copra oil (Sigma Chemical Co.); (*6*) *Soybean oil.*—Sigma Chemical Co.; (*7*) *Sucrose.*—>99.5% (Sigma Chemical Co.); (*8*) *Potassium chloride.*—Crystalline (Sigma Chemical Co.); (*9*) *Citric acid.*—Tripotassium salt (Sigma Chemical Co.); (*10*) *Magnesium phosphate.*—Dibasic trihydrate (Sigma Chemical Co.); (*11*) *Potassium hydroxide.*—ACS reagent (Sigma Chemical Co.); (*12*) *Choline bitartrate.*—Sigma Chemical Co.; (*13*) *L-carnitine.*—Hydrochloride, synthetic (Sigma Chemical Co.); (*14*) *Safflower oil.*—From *Carthamus tinctorius* seed (Sigma Chemical Co.); (*15*) *Ferrous sulfate.*—Heptahydrate (Sigma Chemical Co.); (*16*) *Zinc sulfate.*—Heptahydrate (Sigma Chemical Co.); (*17*) *Manganese sulfate.*—Monohydrate (Sigma Chemical Co.); (*18*) *Sodium citrate.*—Trisodium salt, dihydrate (Sigma Chemical Co.); (*19*) *Lecithin.*—Soy, refined (ICN Biomedicals, Inc., Costa Mesa, CA); (*20*) *Soy protein isolate.*—Protein 92% (ICN Biomedicals, Inc.); (*21*) *Palm olein.*—Fully refined (Fuji Vegetable Oil, Inc., Savannah, GA); (*22*) *Instant nonfat dry milk.*—Spray process (Land O'Lakes, Inc., Minneapolis, MN); (*23*) *Whey protein concentrate.*—34% Protein (Land O'Lakes); (*24*) *Calcium citrate.*—Tetrahydrate (Alfa Aesar Organics, Ward Hill, MA); (*25*) *Inositol.*—Aldrich Chemical Co., Milwaukee, WI; and (*26*) *Maltodextrin.*—Dextrose equivalent 16.5–19.5 (Aldrich Chemical Co.).

Chromatographic Conditions

(a) *Instrument parameters.*—Injection volume, 50 μL; flow rate, 1.0 mL/min; fluorescence detector parameters for all-rac-α-tocopheryl acetate and tocopherols (exλ = 285 nm, emλ = 310 nm, gain = 6); fluorescence detector parameters for retinyl palmitate (exλ = 325 nm, emλ = 470 nm, gain = 9).

(b) *LC configuration.*—Inject sample and standards for all-rac-α-tocopheryl acetate analysis first. Use run time of 20 min to allow all tocopherols to elute. Upon completion of all-rac-α-tocopheryl acetate analysis, change to mobile phase for retinyl palmitate and allow 30 min to equilibrate. Inject standards and samples using run time of 10 min.

Sample Preparation and Description

The ZRMs for milk and soy-based infant formula were manufactured at the Food Process Research and Development Laboratory, Food Science and Technology Department, UGA, Athens, GA. The formulations and ingredients used in this study closely approximate commercial batch records but the exact formulations are confidential. Our manufactured ZRMs closely mimic commercially available milk- and soy-based infant formula, and the ingredient composition is similar to commercial formula labeling. For the soy-based formula, ca 510 g soy protein, 929 g total fat, 1300 g maltodextrin, and various salts were mixed with 10 000 g water and spray-dried to yield ca 5 lb dry formula. The milk-based formula was similarly made but lacked soy protein.

Weigh each of the ingredients into large container and mix with water without oil components. After mixture is lump free and free flowing, add oil components to mixture. Continue mixing while adding more water. Continue to mix for ca 30 min. Pass each formula (milk- and soy-based) through homogenizer (single stage) at 6000 psi. Following homogenization, pasteurize the preparation at temperature of 225°C with 5 s hold time. Spray-dry pasteurized preparation with inlet temperature of 275°C and outlet temperature of 115°C. During spray-drying, continue to mix infant formula on Vortex mixer. Place ca 35–45 g resultant spray-dried

powder into 125 mL sample bottles, blanket the powder with nitrogen and freeze at −20°C until needed.

Characterization

(a) *Retinyl palmitate and all-rac-α-tocopheryl acetate (2).*—Reconstitute ZRM by adding 50 g boiling water to 10 g powder with thorough mixing. Accurately weigh ca 6.5 g reconstituted ZRM into 100 mL low-form glass cylinder (Fisherbrand No. 08-530C). Add 10 mL boiling isopropanol, mix, and add 7.5 g $MgSO_4$. Thoroughly mix with stainless steel spatula. Add 25 mL extraction solution, 1 mL BHT solution, and thoroughly mix with spatula.

Homogenize mixture for 1 min at medium speed with the Polytron homogenizer. Rinse generator tip of homogenizer with isopropanol and filter through 60 mL coarse porosity fritted glass filter into 125 mL Philips beaker using vacuum bell jar filtration apparatus. Release vacuum, break up material on fritted glass filter and wash twice with 15 mL extraction solution.

Repeat extraction by transferring material on fritted glass filter to original glass cylinder. Add 20 mL extraction solution, 5 mL isopropanol, and homogenize mixture for 1 min with Polytron homogenizer. Repeat procedure in preceding paragraph beginning with "Rinse the generator tip...."

Transfer combined filtrate to 500 mL round bottom flask, add 0.5 g $MgSO_4$, and evaporate to dryness with rotary evaporator. Add 15 mL hexane to residue and filter through 0.45 μm nylon filter into 125 mL Philips beaker using vacuum bell jar filtration apparatus. Rinse flask and wash filter 3 times with 7 mL portions of hexane.

Transfer filtrate to 200 mL turbovap flask and evaporate to 1 mL with nitrogen pressure of 8 psi and water bath temperature of 45°C. Quantitatively transfer the final 1 mL extract to 10.0 mL volumetric flask and dilute to volume with hexane.

For all-rac-α-tocopheryl acetate determination, inject 50 μL of extract in conjunction with all-rac-α-tocopheryl acetate standards, using all-rac-α-tocopheryl acetate mobile phase and fluorescence parameters. For retinyl palmitate determination, inject 50 μL extract in conjunction with standards, using mobile phase and fluorescence parameters for retinyl palmitate.

(b) *Protein.*—Proximate analysis method for milk-based infant formula (3).

(c) *Calories.*—Calories were determined by Parr Bomb.

(d) *Ash.*—Proximate analysis method for milk-based infant formula (3).

(e) *Moisture.*—Proximate analysis method for milk-based infant formula (3).

(f) *Riboflavin.*—Fluorometric method (4).

(g) *Thiamin.*—Fluorometric method (5).

(h) *Fat.*—Proximate analysis method for milk-based infant formula (3).

Results and Discussion

ZRMs for fabricated foods such as infant formula can be easily manufactured. Published information as well as label ingredients allow quick determination of the product's composition. One can obtain from speciality suppliers all ingredients necessary to formulate and make product ZRMs devoid of specific micronutrients if these micornutrients are present only as an added fortification premix. In some cases endogenous micronutrients may need to be stripped from the raw ingredient in order to generate a true ZRM for that

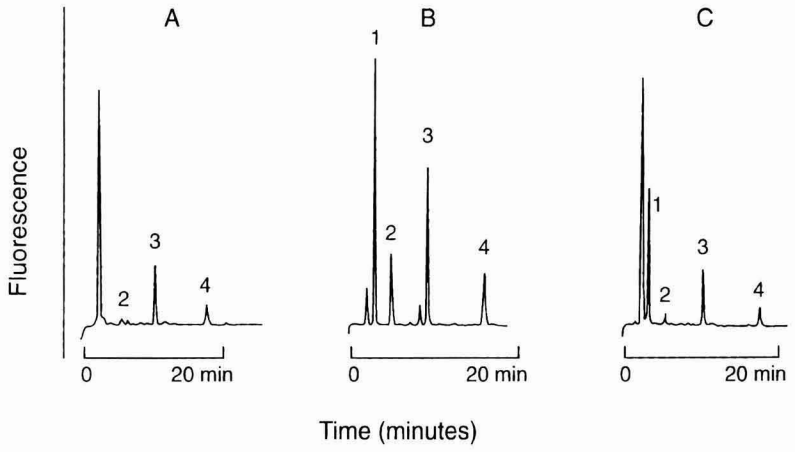

Figure 1. Liquid chromatograms of vitamin E using fluorescence detection (ex λ = 285 nm, em λ = 310 nm); flow rate, 1.0 mL/min; injection volume, 50 μL; mobile phase, 0.5% isopropanol in hexane. A, chromatogram of unfortified zero control extract; B, chromatogram of standard; C, chromatogram of spiked zero control equivalent to 133 μg/g. All-rac-α-tocopheryl acetate is identified as peak (1); RRR-α-tocopherol is identified as peak (2); γ-tocopherol is identified as peak (3); δ-tocopherol is identified as peak (4).

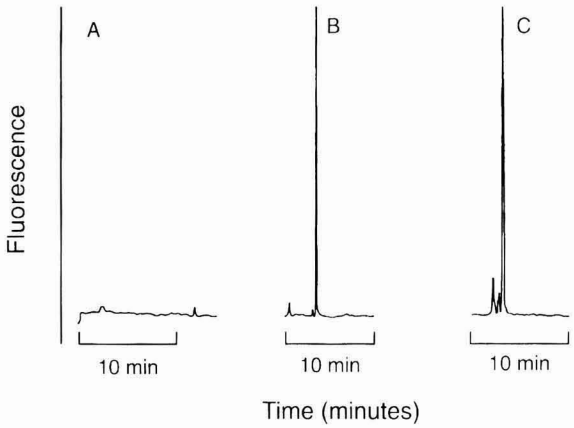

Figure 2. Liquid chromatogram of retinyl palmitate using fluorescence detection (ex λ = 325 nm, em λ = 470 nm); flow rate, 1.0 mL/min; injection volume, 50 μL; mobile phase, 0.125% isopropanol in hexane. A, chromatogram of unfortified zero control extract; B, chromatogram of retinyl palmitate standard; C, chromatogram of spiked zero control equivalent to 27.5 μg/g. Retinyl palmitate is identified as the major peak in the chromatograms.

specific nutrient. In the case of infant formula, micronutrients are typically added as a fortification premix. Thus, in order to manufacture infant formula ZRMs, it was a simple matter of eliminating the fortification premix. We successfully generated a ZRM for dairy and nondairy infant formula without all-rac-α-tocopheryl acetate and retinyl palmitate. These ZRMs are available for method validation studies.

Figure 1 illustrates the chromatogram for all-rac-α-tocopherol acetate in the soy-based infant formula ZRM, where A is the chromatogram for the ZRM, B is a chromatogram of the all-rac-α-tocopherol acetate standard and naturally occurring tocopherols, and C is the ZRM spiked with all-rac-α-tocopherol acetate equivalent to 133 μg/g. Figure 2 shows the chromatogram for retinyl palmitate in the soy-based infant formula ZRM, where A is the ZRM, B is the retinyl palmitate standard, and C is the ZRM spiked with retinyl palmitate equivalent to 27.5 μg/g. Chromatograms of ZRM extracts clearly show that the product has nondetectable levels of retinyl palmitate and all-rac-α-tocopheryl acetate, thus allowing the use of these ZRMs in method development and validation studies where one would fortify the ZRM with the analyte of interest. In addition, the ZRMs were characterized for proximates, fatty acid content, and some water soluble vitamins. The compositions are shown in Table 1. The protein and fat contents meet the Code of Federal Regulations requirements for nutrients in infant formula. The moisture and ash levels represent levels normally found in powdered infant formula samples that have been routinely analyzed in this laboratory. Furthermore, each ZRM was assayed for thiamin and riboflavin. The soy ZRM contained no thiamin or riboflavin above a screening level of 12.1 and 2.36 μg/100 kcal, respectively; however, the milk ZRM contained 50 μg/100 kcal thiamin and 110 μg/100 kcal riboflavin. The thiamin and riboflavin found in the milk ZRM can be attributed to the nonfat dry milk used in the manufacturing process.

Table 1. Characterization data for infant formula ZRM

Component	Milk ZRM	Soy ZRM	Replicates (*n*)
Protein (g/100 kcal)	2.11 ± 0.23	2.41 ± 0.60	3
Moisture (%)	1.72 ± 0.06	0.464 ± 0.03	3
Ash (%)	3.22 ± 0.02	2.18 ± 0.10	3
Fat (g/100 kcal)	4.83	4.23	1
Calories (kcal/serving)	113	116	1

The ZRMs as described can be used as a control to ascertain method performance for retinyl palmitate and all-rac-α-tocopheryl acetate analysis in infant formula at levels <100% of the label claim. Ultimately, such a ZRM will simplify methods development for these vitamins in infant formula. The ZRM can also be customized to suit one's needs by varying either the type of oil, protein, or both in the formulation. ZRMs for other fabricated products will enhance analytical methods development for a host of micronutrients.

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RESIDUES AND TRACE ELEMENTS

Optimization of Selenium Determination in Human Milk and Whole Blood by Flow Injection Hydride Atomic Absorption Spectrometry

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A flow injection hydride atomic absorption spectrometric (FI-HAAS) method was developed for determining selenium in human milk and whole blood after microwave digestion of the sample. The sample (2 mL human milk or 0.25 mL blood) was introduced into the microwave vessel with 1.5 mL HNO_3 and 0.25 mL H_2O_2 and 300 W (4 min) and 600 W (4 min) were applied. The digestion was completed by heating to 140°C (2–3 h). Se (VI) was reduced to Se (IV) with hydrochloric acid. The instrumental conditions for FI-HAAS (concentrations of reducing agent and carrier acid, flow rate of argon carrier gas, and sample volume injected) were optimized. The detection limit of the proposed method was 0.23 ng/mL (assay) or 115 pg Se (absolute) in biological samples (1.15 ng/mL milk, 10.4 ng/mL blood). The precision values were 5.0% for milk and 4.0% for blood. The accuracy was evaluated with 2 reference materials, National Institute of Standards and Technology Non-Fat Milk Powder (found: 104.3 ± 7.2 ng/g, certified: 110 ± 10 ng/g) and Whole Blood Seronorm (found: 81 ± 7.3 ng/mL, reference: 83 ± 4 ng/mL). The results show the suitability of the method for selenium determination in human milk and whole blood. The method was applied to whole blood samples obtained from pregnant women and to human milk.

Measuring the essential trace element selenium in biological fluids is an important first step in estimating selenium nutritional status that requires accurate and sensitive techniques (1).

We previously described a spectrofluorimetric method (2) for selenium determination in human milk, that meets the requirement of high sensitivity imposed by the low selenium content of milk. Although digestion time was reduced by using microwave digestion, the method is long and laborious. It is necessary to eliminate the residues of organic matter and of the nitric acid used to digest the sample, which can interfere in the fluorimetric determination. Therefore, the piazselenol complex to be measured must be extracted.

The hydride generation technique combined with atomic absorption spectrometry (HG-AAS) offers the advantage of high sensitivity, together with reduction of matrix interference. Determination of the selenium content of biological fluids by this technique also requires destruction of the organic matter and a complete reduction of Se(VI) to Se(IV). Moreover, the method has to be optimized for each type of sample. Some authors have used an acid mixture for digestion of human blood plasma or serum: a digestion mixture consisting of HNO_3–H_2SO_4–$HClO_4$ (5 + 2 + 1) was used by McLaughlin et al. (3), who obtained recoveries between 95 and 109%. They did not found any appreciable matrix effect in selenium determination in serum or plasma by flow injection hydride atomic absorption spectrometry (FI-HAAS). Negretti de Brätter et al. (4) obtained good accuracy and precision by digestion (HNO_3–H_2SO_4–$HClO_4$) in an open vessel or in a pressurized vessel when reference materials of tissues or serum were analyzed by FI-HAAS. Mayer et al. (5) obtained good accuracy for selenium determination in serum after predigestion with HNO_3–H_2SO_4, followed by microwave digestion and HG-AAS measurement. Wang et al. (6) used 10 mL whole blood for Fe, Zn, Cu, and Se determination after HNO_3–H_2SO_4–$HClO_4$ digestion; the recovery obtained for selenium in serum was only 84% by hydride generation flame AAS. The authors attribute this to selenium volatility; also, apparently, there was matrix interference in the whole-blood selenium determination. Ducros et al. (7) obtained good results, using microwave digestion in an open-vessel system and heating under reflux with HNO_3–H_2O_2, with determination of selenium in whole-blood by gas chromatography/mass spectrometry. Finally, Navarro et al. (8) and Hao et al. (9), using HNO_3–$HClO_4$, obtained better recoveries (104 and 97–99.2%, respectively) from urine and serum with HG-AAS determination.

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Given the fact that few studies have determined selenium concentration in whole blood, which is a more complex matrix than serum or plasma, and because this information would be useful in determining selenium nutritional status, the present study was designed to develop an FI-HAAS method for determining selenium in whole blood and human milk after microwave digestion of the samples. Our aim was to use this method to evaluate the selenium nutritional status of pregnant women (whose selenium values might perhaps be lower than those of nonpregnant women [10]), and also to estimate the selenium intake of breast-fed infants. After the analytical parameters of the method were determined, the method was applied to the blood of pregnant women and to human milk to test its usefulness.

Experimental

Apparatus

(a) *Spectrophotometer.*—Perkin-Elmer Model 2380 with Perkin-Elmer selenium electrodeless discharge lamp and EDL System 2 power supply (Perkin-Elmer, Norwalk, CT).

(b) *Flow injection analysis system.*—Perkin-Elmer MHS-FIAS 100 with manifold and separator blocks to separate the gas–liquid mixture.

(c) *Quartz absorption cell.*—Heated by air–acetylene flame (Perkin-Elmer).

(d) *Microwave oven.*—Milestone MLS 1200 digestion system with bomb, medium-pressure Teflon vessels MV 100 with burst disc.

(e) *Block digestion system.*—Kjeldatherm Gerhardt block digestor.

Reagents

All reagents were analytical reagent grade. A Millipore Milli-Q Plus deionized water system (Waters, Millipore, Medford, MA) was used throughout.

(a) *Hydrochloric acid.*—37%, sp. gr. 1.33 (Probus, Badalona, Spain).

(b) *Nitric acid.*—65%, sp. gr. 1.40 (Merck, Darmstadt, Germany).

(c) *Sodium tetrahydroborate.*—98–99% (Sigma, St. Louis, MO).

(d) *Sodium hydroxide.*—97% (Panreac, Barcelona, Spain).

(e) *Hydrogen peroxide.*—33% (Panreac).

(f) *Selenium standard solutions.*—(*1*) *Stock solution.*—1 g/L, prepared from an ampoule of selenium dioxide in dilute nitric acid (Titrisol, E. Merck). (*2*) *Intermediate solutions.*—10 μg/mL (0.100 mL stock solution diluted to 10 mL with deionized water). (*3*) *Standard working solutions.*—Prepared from the stock solution immediately before use.

All glassware was soaked in concentrated nitric acid for 10 min and rinsed with deionized water before use.

Samples

Breast milk samples (colostrum) from healthy mothers were collected directly in clean glass bottles by using a breast pump.

Blood samples were obtained by venipuncture from healthy pregnant volunteers in different months of gestation. The blood samples were collected in plastic tubes, and 1 drop of heparin sodium anticoagulant solution was added to each 5 mL blood sample.

All breast milk and blood samples were provided by the University Hospital "La Fe."

Biological reference materials (Non-Fat Milk Powder National Institute of Standards and Technology Standard Reference Material [NIST SRM] 1549 and Whole Blood I Seronorm Trace Elements) were also used.

Digestion

Each sample (2 mL human milk or 0.25 mL blood) was introduced into a microwave vessel, and 1.5 mL HNO_3 and 0.25 mL H_2O_2 were added (2). The microwave digestion program was applied: 300 W (4 min) followed immediately by 600 W (4 min). The digested samples were transferred to digestion tubes by using 4 mL water to wash each Teflon vessel. The tubes were then heated to 140°C for 2–3 h to reduce the volume to 1 mL and to obtain a clear digest. After cooling, 2 mL HCl was added to reduce Se(VI) to Se(IV). The volume was then adjusted to 10 mL with deionized water, and the tubes were stoppered and placed in a water bath at 100°C for 10 min.

The digestion procedure should also be applied to the standard working solutions, or at least 2 mL HCl should be added before determination.

Determination

Selenium was determined under the instrumental conditions described in Table 1, which were optimized by conducting assays with various concentrations of the reducing agent, various concentrations of HCl in the carrier stream, and various flow rates of the argon carrier gas.

Results and Discussion

Optimization of Analytical Conditions

Peak height increased with increase in sample volume. Although loops up to 1 mL were assayed, a loop of 0.5 mL was chosen to conserve sample.

In the trials carried out with standards, HCl carrier concentrations of 1.5–3% in the argon carrier gas were

Table 1. Instrumental parameters for selenium determination

Atomic absorption spectrophotometric conditions[a]
AAS 2380 (Perkin-Elmer):
Wavelength (nm): 196.0
Slit width (nm): 2.0
Light source: electrodeless discharge lamp (Se)
power supply (mA): 280
Flame, flow setting (L/min): air (17), acetylene (1.5)
Integration time (s): 15
Flow injection analysis conditions
MHS- FIAS 100 (Perkin-Elmer):
Time setting (s) fill: 10
inject: 15
Carrier acid: 3% HCl, flow rate (mL/min): 9
Reductant: 0.05% $NaBH_4$ in 0.1% NaOH,
flow rate (mL/min): 4
Argon gas flow (mL/min): 100
Sample volume (μL): 500

[a] For measurement of peak height.

needed to obtain a maximum response. Therefore 3% HCl was used to ensure that the HCl concentration of the carrier was sufficient.

Assays with solutions of 0.05, 0.1, and 0.2% sodium tetrahydroborate, the reductant, were conducted, and the 0.05% concentration was chosen, because it produced the greatest sensitivity, as shown by the slopes of the calibration curves in Figure 1.

Assays were also conducted to determine the influence of the argon flow rate on the signal. A plot of absorbance vs flow rate is shown in Figure 2. A flow rate of 100 mL/min was chosen.

To study the influence of the matrix, the method of standard additions was applied to the analysis of milk (Figure 3) and blood samples (Figure 4). An analysis of

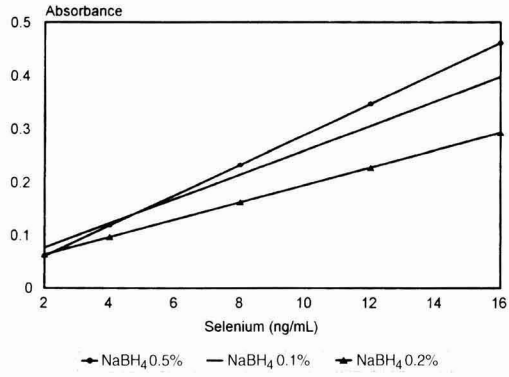
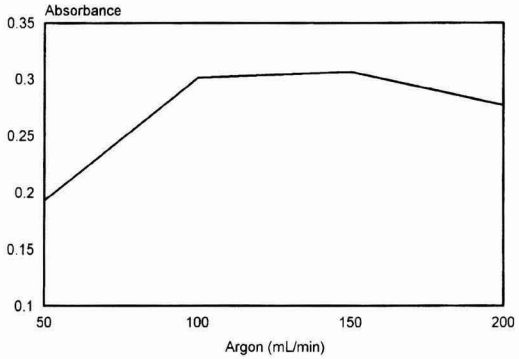


Figure 1. Optimization of tetrahydroborate concentration for FI-HAAS determination of selenium.



Se: 10 ppb HCl: 3% $NaBH_4$: 0.2%

Figure 2. Optimization of argon flow rate for FI-HAAS determination of selenium.

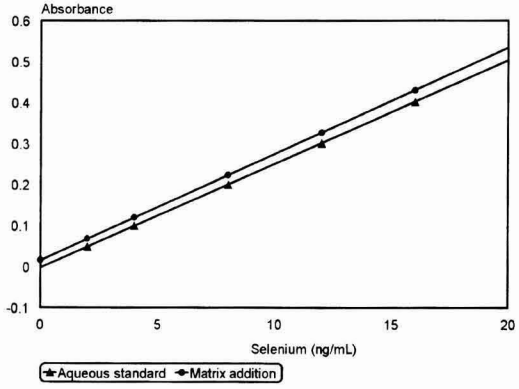


Figure 3. Assay results for matrix interference in the FI-HAAS determination of selenium in human milk.

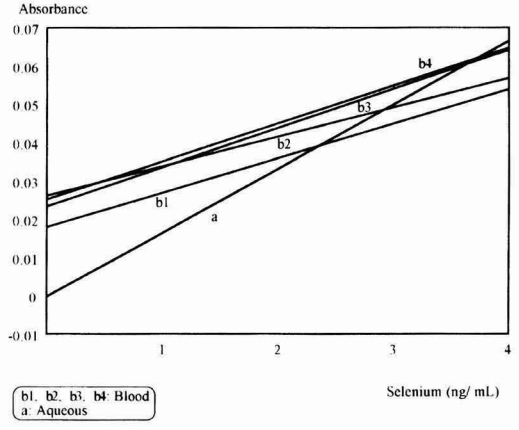


Figure 4. Assay results for matrix interference in the FI-HAAS determination of selenium in different blood samples.

covariance was performed, and no matrix interference was found in the analysis of the milk samples ($F_{calculated} = 1.24$, $F_{table}^{0.05} = 5.32$); however, a matrix interference, at a probability level of 95%, was observed in the determination of selenium in blood ($F_{calculated} = 411.71$, $F_{table} = 7.71$). Therefore, it is necessary to apply the method of standard additions when the selenium content of blood is measured.

To determine whether it is possible to use the same calibration curve to calculate the selenium content of different blood samples, blood samples from 4 different individuals were analyzed by the method of standard additions (Figure 4), and the slopes of the curves obtained were compared (analysis of covariance). No differences at a probability level of 95% were observed ($F_{calculated}$: $b_1 - b_2 = 0.004$, $b_1 - b_3 = 4.9$, $b_1 - b_4 = 2.4$, $b_2 - b_3 = 4.5$, $b_2 - b_4 = 1.9$, $b_3 - b_4 = 3.6$, where b values with different subscripts correspond to different blood samples; $F_{table} = 7.71$). We therefore concluded that a blood pool (a mixture of the samples to be analyzed) can be used to obtain a single calibration curve for each assay, when selenium is determined in different blood samples.

The optimal conditions selected for selenium determination are summarized in Table 1.

Analytical Characteristics

To assess the validity of the proposed method, the detection limit, the precision, and the accuracy of the method were estimated. Linearity of response was verified by using standards ranging from 1 to 30 ng/mL. The adjusted linear equations and correlation coefficients obtained were as folllows: for milk, $y = 0.03x + 0.04$, r = 0.9968; for blood, $y = 0.0148x - 0.00044$, r = 0.9997 for blood samples.

The detection limit, defined as the selenium concentration corresponding to 3 times the standard deviation of the reagent blanks, was calculated with 10 reagent blanks, which were subjected to the same digestion procedure as the samples. The value of the detection limit was calculated with respect to the assay and to the sample (Table 2). These values are comparable with those reported in the literature; the absolute determination limit in biological samples was 115 pg, whereas 110 pg has been reported for human body fluids (4).

The instrumental precision was estimated from 10 consecutive measurements of the same dilution of a digested sample (blood and milk, respectively). The precision of the method was estimated from analyses of 16 homogeneous aliquots of the sample (involving all sample pretreatment steps) performed on 2 different days (within-run and between-run precision). The results, expressed as relative standard deviations (RSDs) are shown in Table 2.

The accuracy was estimated by measuring the selenium content of biological reference standards (NIST and Seronorm). The results obtained show good agreement with the certified values (Table 2).

Table 2. Analytical parameters for determination of selenium in milk and blood

Parameter	Blood	Milk
Detection limit:	0.26 ng/mL[a]	0.23 ng/mL[a]
(n = 10)	10.4 ng/mL[b]	1.15 ng/mL[b]
Matrix effect	Yes	No
Precision (%RSD)		
Instrumental (n = 10)	4.5 (x = 62.0 ng/mL)	3.2 (x = 7.8 ng/mL)
Method		
Within-run (n = 8)	4.0 (x = 66.8 ng/mL)	5.0 (x = 22.5 ng/mL)
Between-run (n = 16)	10.7	7.1
Accuracy	83 ± 4 ng/mL[c]	110 ± 10 ng/g[d]
Certified value for Se	81 ± 7.3 ng/mL[c]	104.3 ± 7.2 ng/g[d]

[a] With respect to the assay.
[b] With respect to the sample.
[c] Whole Blood reference material: Seronorm batch 205052.
[d] Non-Fat Milk Powder reference material: NIST SRM 1549.

Table 3. Selenium concentrations found in human milk and whole blood

Sample	n	Mean ± SD, μg/L	95% confidence interval for women: Minimum, μg/L	Maximum, μg/L
Whole blood[a]	54	74.67 ± 18.74	69.54	79.79
Human milk	37	9.60 ± 4.43	8.11	11.09

[a] Obtained from pregnant women.

Analysis of Samples

The proposed method was applied to 37 human milk samples and 54 whole blood samples obtained from pregnant women. The analytical results are shown in Table 3.

Conclusions

The detection limits, the linear range of calibration, and the precision and accuracy values obtained for the proposed method, and the analytical results obtained with the proposed method demonstrate its suitability for determining selenium in human whole blood of pregnant women and in human milk.

Acknowledgments

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RESIDUES AND TRACE ELEMENTS

Stability of Organic Pollutants During Microwave-Assisted Extraction from Solid Matrixes

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A stability study was conducted for 95 semivolatile organics listed in U.S. Environmental Protection Agency Method 8250 (this number includes 6 surrogate compounds). These compounds were spiked into solvent only [hexane–acetone (1 + 1), methylene chloride–acetone (1 + 1), toluene–methanol (10 + 1), and methyl *tert*-butyl ether], solvent/dry soil suspensions, and solvent/wet soil suspensions [20% water (w/w)] and heated with microwave energy in closed vessels at 50° or 145°C for 5 or 20 min. For comparison and to determine nitrogen blowdown losses, spiked solvent samples that had not been exposed to microwave energy were concentrated by the blowdown technique and analyzed for each of the spiked compounds. Hexane–acetone (1 + 1) seems to be the best for the compounds and matrixes investigated, with recoveries > 80%, except for basic compounds and benzoic acid in the solvent/dry soil suspension experiments. Increasing extraction time from 5 to 20 min did not increase recoveries; in fact, recoveries of neutral compounds decreased slightly at the longer extraction time. Increasing the temperature from 50° to 145°C decreased recoveries of basic compounds by about 10%. Recoveries of basic compounds, of benzoic acid (and probably other organic acids as well), and at least to some extent of phenolic compounds from the solvent/wet soil suspensions were higher than those for solvent/dry soil suspensions.

In earlier studies (1, 2) we reported on the extractability under microwave-assisted extraction (MAE) conditions of 95 compounds listed in U.S. Environmental Protection Agency (EPA) Method 8250, 45 organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) listed in EPA Method 8081, and 47 organophosphorus pesticides listed in EPA Method 8141 from freshly spiked soil samples; spiked soil samples that had been aged for 24 h, 14 days, or 21 days; and a few standard reference materials. For these extractions, we used hexane–acetone (1 + 1) at 115°C for 10 min.

Solvents other than hexane–acetone have been used for more polar compounds (3); however, none of the solvents recommended by EPA in Methods 3540 and 3550 (e.g., methylene chloride–acetone and toluene–methanol) has been evaluated thoroughly for use under MAE conditions. Furthermore, degradation or conversion of compounds that may occur when using microwave energy to heat the solvent/soil suspension has not been investigated. Possible ways in which compound degradation may occur include exposure to temperature and pressure inside the microwave extraction vessel, interaction with other analytes or with solvent under these conditions, and catalysis by the matrix. To determine whether degradation under MAE conditions presents a problem, a stability study was conducted for 95 semivolatile organics listed in EPA Method 8250 (this number includes 6 surrogate compounds). These compounds were spiked into solvent only (hexane–acetone, 1 + 1; methylene chloride–acetone, 1 + 1; toluene–methanol, 10 + 1; and methyl *tert*-butyl ether), solvent/dry soil suspensions, and solvent/wet soil suspensions (20% water, w/w) and heated in closed vessels with microwave energy at 2 temperatures (50° or 145°C) for 5 or 20 min. For comparison and to determine nitrogen blowdown losses, spiked solvent samples that had not been exposed to microwave energy were concentrated by the blowdown technique and analyzed for each of the spiked compounds. Recoveries reported here have been corrected for blowdown losses.

Experimental

Standards

Analytical reference standards of the 95 compounds were purchased from Absolute Standards, Inc. (Camden, CT) as 8 composite solutions in methylene chloride (mix 1 consisting of 14 ethers, phthalates, and nitrosamines; mix 2 of 14 compounds, mostly of chlorinated benzenes, nitrobenzene, and nitrotoluenes; mix 4

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of 3 phenols and benzoic acid; mix 5 of various anilines, dibenzofuran, benzyl alcohol, and 2-methylnaphthalene; mix 8 of 13 phenols; mix 9 of 8 miscellaneous compounds; mix 10 of ethyl methanesulfonate and methyl methanesulfonate; and mix 11 of 11 nitrogen-containing compounds), one composite solution in methanol (mix 6 consisting of benzidine and 3,3′-dichlorobenzidine), and one composite solution in methylene chloride–benzene (1 + 1) consisting of 17 polynuclear aromatic hydrocarbons. In these mixtures, the concentrations of each compound was 2 mg/mL. Dibenzo(*a,j*)acridine was purchased from Chem Service (West Chester, PA) and 1,2-diphenylhydrazine from Aldrich Chemical (Milwaukee, WI). The 6 surrogate compounds listed in Table 1 were purchased from Absolute Standards as 2 composite solutions: the acid surrogate standard contained 2-fluorophenol, phenol-d_5, and 2,4,6-tribromophenol at 2 mg/mL in methanol, and the base/neutral surrogate standard contained 2-fluorobiphenyl, terphenyl-d_{14}, and nitrobenzene-d_5 at 1 mg/mL in methylene chloride. Six internal standards (1,4-dichlorobenzene-d_4, naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12}) were purchased from Supelco, Inc. (Bellefonte, PA) as a composite solution at 2 mg/mL in methylene chloride; their purities were stated to be 99%. An intermediate stock solution of all target compounds at 125 μg/mL (except for 2 fluorobiphenyl, nitrobenzene-d_5, and terphenyl-d_{14} at 62.5 μg/mL) was prepared by combining calculated amounts of the various composite solutions and diluting to volume with methylene chloride. Calibration standards at 5, 10, 15, and 50 μg/mL were prepared by serial dilution with methylene chloride of the 125 μg/mL composite solution. The 6 internal standards were spiked into every calibration standard and sample extract at 40 μg/mL.

Soil

Soil was obtained from Sandoz Crop Protection (Gilroy, CA); its reported characteristics are pH, 7.5; cation exchange capacity, 14.6 mequiv/100 g; organic carbon content, 0.1%; water content, 2.6%; sand, 57.6%, silt, 21.8%; and clay, 20.6%.

Solvents

Solvents were distilled-in-glass and pesticide grade and were obtained from Baxter Scientific (McGaw Park, IL).

Procedure for MAE

MAEs were performed with a MES-1000 microwave sample extraction system (CEM Corporation, Matthews, NC), described in reference 1.

Extraction was as follows: a 5 g portion of soil was accurately weighed in an aluminum dish and transferred quantitatively to the Teflon-lined extraction vessel. To prepare wet samples, the calculated volume of water was added to the sample in the extraction vessel and allowed to equilibrate with the matrix for ca 10 min. A solution containing the test compounds and the 6 surrogate compounds was added to each sample immediately before solvent (30 mL) was added. After ensuring that a new rupture membrane was in place, the extraction vessel was closed. Extractions were performed at 50° or 145°C for 5 or 20 min at 100% power. After extraction, vessels were allowed to cool to room temperature for ca 20 min before they were opened. The supernatant was filtered through glass wool prewashed with solvent and then combined with the 2 to 3 mL solvent rinse of the residue. The extract was concentrated to 1 mL by nitrogen blowdown for analysis by gas chromatography/mass spectrometry (GC/MS). Four solvents, namely 1 + 1 hexane–acetone (HA), 1 + 1 methylene chloride-acetone (MA), 10 + 1 toluene–methanol (MT), and methyl *tert*-butyl ether (MB), were used.

Analysis of Extract

Analyses were performed with a Hewlett-Packard 5890 Series II gas chromatograph interfaced with a Hewlett-Packard 5971A mass spectrometer MSD/DOS Chemstation (Hewlett Packard, Palo Alto, CA) and equipped with a Hewlett-Packard 5973A autoinjector. Samples were introduced via a 30 m length × 0.25 mm id × 0.25 μm film thickness DB-5 fused-silica open-tubular column (J & W Scientific, Folsom, CA) with helium as carrier gas at a flow rate of ca 1 mL/min. Column temperature was held at 40°C for 4 min and then increased at 8°C/min to a final temperature of 300°C, where it was held for 10 min. Injection volume was 1 μL, and injector temperature was 250°C. Injector was set in splitless mode for 1 min after injection. Electron energy was set at 70 eV, and electron multiplier voltage at 2160 V. Data were acquired at 1 s/scan (scanning range was 35–500 amu). The instrument was tuned daily with decafluorotriphenyl phosphine (DFTPP) introduced via the gas chromatograph inlet. A 5-point internal standard calibration using standards at 5, 10, 25, 50, and 100 μg/mL was performed daily to establish the GC/MS linear range. Six internal standards were spiked into every calibration standard and sample extract that was analyzed by GC/MS. For quantitation, we used average relative response factors from multilevel calibration.

Safety

The microwave unit, which incorporates several safety features described in reference 1, must be operated in accordance with the manufacturer's recommended operating safety instructions. A new rupture membrane per vessel should be used for each extraction. Should the membrane rupture because of in-

Table 1. Compounds investigated in this study

Group 1: Neutral Compounds

1. Methyl methanesulfonate
2. Ethyl methanesulfonate
3. Bis(2-chloroethyl)ether
4. 1,3-Dichlorobenzene
5. 1,4-Dichlorobenzene
6. 1,2-Dichlorobenzene
7. Benzyl alcohol
8. Bis(2-chloroisopropyl)ether
9. Hexachloroethane
10. *N*-Nitroso-dipropylamine
11. Nitrobenzene-d_5[a]
12. Acetophenone
13. Nitrobenzene
14. Isophorone
15. Bis(2-chloroethoxy)methane
16. 1,2,4-Trichlorobenzene
17. Hexachlorobutadiene
18. 1,2,4,5-Tetrachlorobenzene
19. Hexachlorocyclopentadiene
20. 2-Fluorobiphenyl[a]
21. Dimethyl phthalate
22. 2,6-Dinitrotoluene
23. Dibenzofuran
24. Pentachlorobenzene
25. 2,4-Dinitrotoluene
26. 2-Chlorophenylphenyl ether
27. Diethyl phthalate
28. 4-Bromophenylphenyl ether
29. Hexachlorobenzene
30. Pentachloronitrobenzene
31. Di-*n*-butyl phthalate
32. Butyl benzyl phthalate
33. Bis(2-ethylhexyl)phthalate
34. Di-*n*-octyl phthalate

Group 2: PAHs

1. Naphthalene
2. 2-Methylnaphthalene
3. 2-Chloronaphthalene
4. 1-Chloronaphthalene
5. Acenaphthylene
6. Acenaphthene
7. Fluorene
8. Phenanthrene
9. Anthracene
10. Fluoranthene
11. Pyrene
12. 4-Terphenyl-d_{14}[a]
13. Benzo(*a*)anthracene
14. Chrysene
15. 7,12-Dimethylbenz(*a*)anthracene
16, 17. Benzo(*b* + *k*)fluoranthene
18. Benzo(a)pyrene
19. 3-Methylcholanthrene
20. Indeno(1,2,3-*cd*)pyrene
21. Dibenzo(a,*h*)anthracene
22. Benzo(*g*,*h*,*i*)perylene

Group 3: Basic Compounds

1. 2-Picoline
2. Aniline
3. *N*-Nitrosopiperidine
4. α,α-Dimethylphenethylamine
5. 4-Chloroaniline
6. *N*-Nitrosodibutylamine
7. 2-Nitroaniline
8. 3-Nitroaniline
9. 1-Naphthylamine
10. 2-Naphthylamine
11. 4-Nitroaniline
12. 1,2-Diphenylhydrazine
13. Phenacetin
14. 4-Aminobiphenyl
15. Pronamide
16. Benzidine
17. *p*-Dimethylaminoazobenzene
18. 3,3′-Dichlorobenzidine
19. Dibenzo(*a*,*j*)acridine

Group 4: Phenolic Compounds

1. 2-Fluorophenol[a]
2. Phenol-d_5[a]
3. 2-Chlorophenol
4. Phenol
5. 2-Methylphenol
6. 4-Methylphenol
7. 2-Nitrophenol
8. 2,4-Dimethylphenol
9. 2,4-Dichlorophenol
10. 2,6-Dichlorophenol
11. 4-Chloro-3-methylphenol
12. 2,4,6-Trichlorophenol
13. 2,4,5-Trichlorophenol
14. 2,4-Dinitrophenol
15. 4-Nitrophenol
16. 2,3,4,6-Tetrachlorophenol
17. 2,4,6-Tribromophenol[a]
18. 4,6-Dinitro-2-methylphenol
19. Pentachlorophenol

Group 5: Acid

1. Benzoic acid

[a] Surrogate compound.

creased pressure inside individual vessels, the solvent vapor is unlikely to leak into the cavity, because all vessels are connected to a containment vessel via the solvent rupture vent tube. To prevent pressure buildup inside individual vessels, wet samples should not be extracted simultaneously with dry samples; when extracting 12 samples simultaneously, they should be either all dry or all wet. Likewise, solvent blanks should not be heated together with samples that are to be extracted by MAE, because the former will heat faster than the latter.

Statistical Analysis

Statistical analyses were performed by using the Statgraphics Plus Version 5 (STSC, Inc., Rockville, MD) commercial software package. For each group of compounds, analysis of variance and multiple comparisons of means were performed at the 95% confidence level.

Results and Discussion

At present, 118 semivolatile compounds are listed in EPA Method 8250 (Revision 1, November 1992). Of these, we initially selected 92 compounds for our experimental work; the other 26 compounds were OCPs and PCBs, which we investigated separately with the compounds listed in Method 8081. From the 92 semivolatile compounds, we are reporting data for only 89 compounds. Benzo(*b*)fluoranthene and benzo(*k*)fluoranthene could not be resolved on the DB-5 column, and we are therefore reporting only one set of numbers for both compounds. We deleted *N*-nitrosodimethylamine, which was difficult to separate from the solvent under the GC conditions used, and *N*-nitrosodiphenylamine, which decomposed in the gas chromatograph inlet to diphenylamine; thus, the latter 2 compounds could not be reliably quantitated by Method 8250 without separate experiments being conducted for each compound. We also investigated 6 surrogate compounds recommended by EPA for use with Method 8250 compounds; thus, the total number of compounds for which we are reporting data is 95.

To facilitate data interpretation, the 95 compounds were divided into 5 groups as follows (Table 1): neutrals (34 compounds), PAHs (22 compounds), basic compounds (19 compounds), phenolic compounds (19 compounds), and acid (1 compound). Recovery data for each compound are included in supplementary material available from the authors.

Neutral Compounds

Figure 1 shows recovery means and 95% confidence intervals for the means for the 34 compounds as a function of matrix [solvent (HA, MA, MT, MB), solvent/dry soil suspension (DS), and solvent/wet soil suspension (WS)], separately for each solvent. Average recoveries (across all 34 compounds) were significantly higher from solvent-alone extracts, followed by the solvent/dry soil and then the solvent/wet soil suspension extracts for all but the MT solvent combination. For MT solvent combination, average recoveries for solvent-alone extracts and solvent/dry soil suspension extracts were not significantly different; however, MT was the only solvent combination of the 4 tested for which average recoveries from the solvent/wet soil suspension extracts were significantly lower than those from the solvent/dry soil suspension extracts.

Figure 2 shows recovery data as a function of time (5 and 20 min), temperature (50° and 145°C) and compound (1 through 34). Raising the temperature from 50° to 145°C resulted in significantly lower overall recoveries, with extraction at 145°C producing lower recoveries. Nonetheless, when recoveries were plotted as functions of compounds, all but 3 compounds (methyl methanesulfonate, ethyl methanesulfonate, and hexachlorocyclopentadiene) had mean recoveries >80%. Mean recoveries of the 3 compounds were 77–79%.

Among 4 solvent combinations, HA appeared to work best for neutral compounds regardless of other factors (soil, time, and temperature).

PAHs

Figures 3 and 4 summarize recovery data for the 22 PAHs. No overall matrix effect could be found (Figure 3), except for MA, for which adding soil (dry or wet) to the solvent significantly reduced average recoveries. However, for this solvent combination, there was no significant difference between recoveries from the solvent/dry soil and the solvent/wet soil suspension extracts. With MB, recoveries were significantly lower from the solvent/wet soil suspensions than from the solvent/dry soil suspensions.

As shown in Figure 4, neither time nor temperature had an overall effect on recoveries. The 4 solvents performed equally well and gave mean recoveries >80%, probably because of the generally high stability of PAHs.

Basic Compounds

Figures 5 and 6 summarize recovery data for the 19 basic compounds. Compared with recoveries of neutral compounds and PAHs, mean recoveries of basic compounds had a wider range, from 95% for MA (solvent only) to 55% for MB/dry soil suspension. Time had no significant effect on recoveries, but temperature did. Average recoveries from samples exposed to 145°C were significantly lower than those from samples exposed to 50°C. Two compounds, α,α-dimethylphenethylamine and benzidine, were most affected by matrix, time, and temperature. For example, benzidine

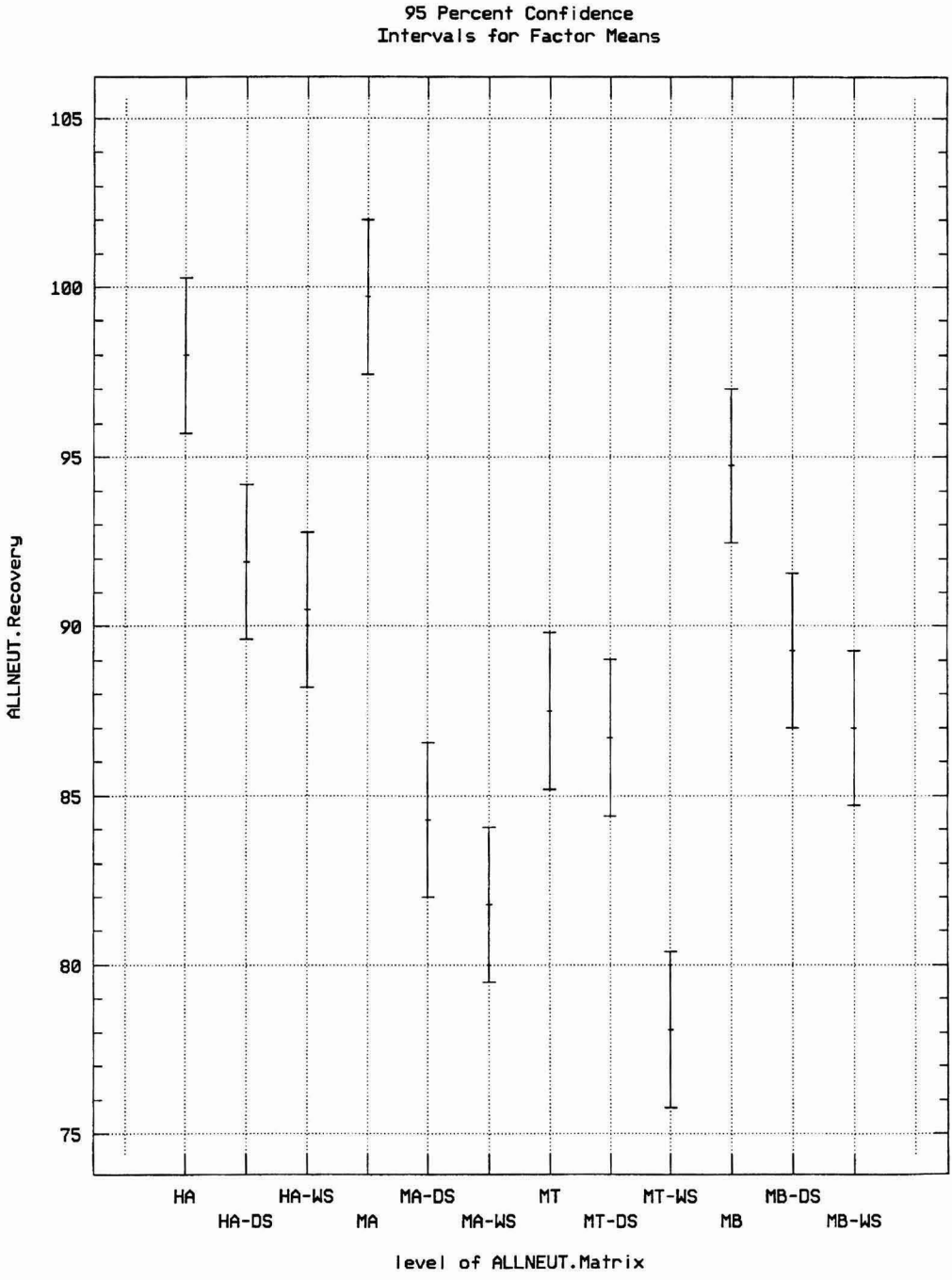

Figure 1. Recovery as a function of matrix for 34 neutral compounds: means and 95% confidence intervals for factor means.

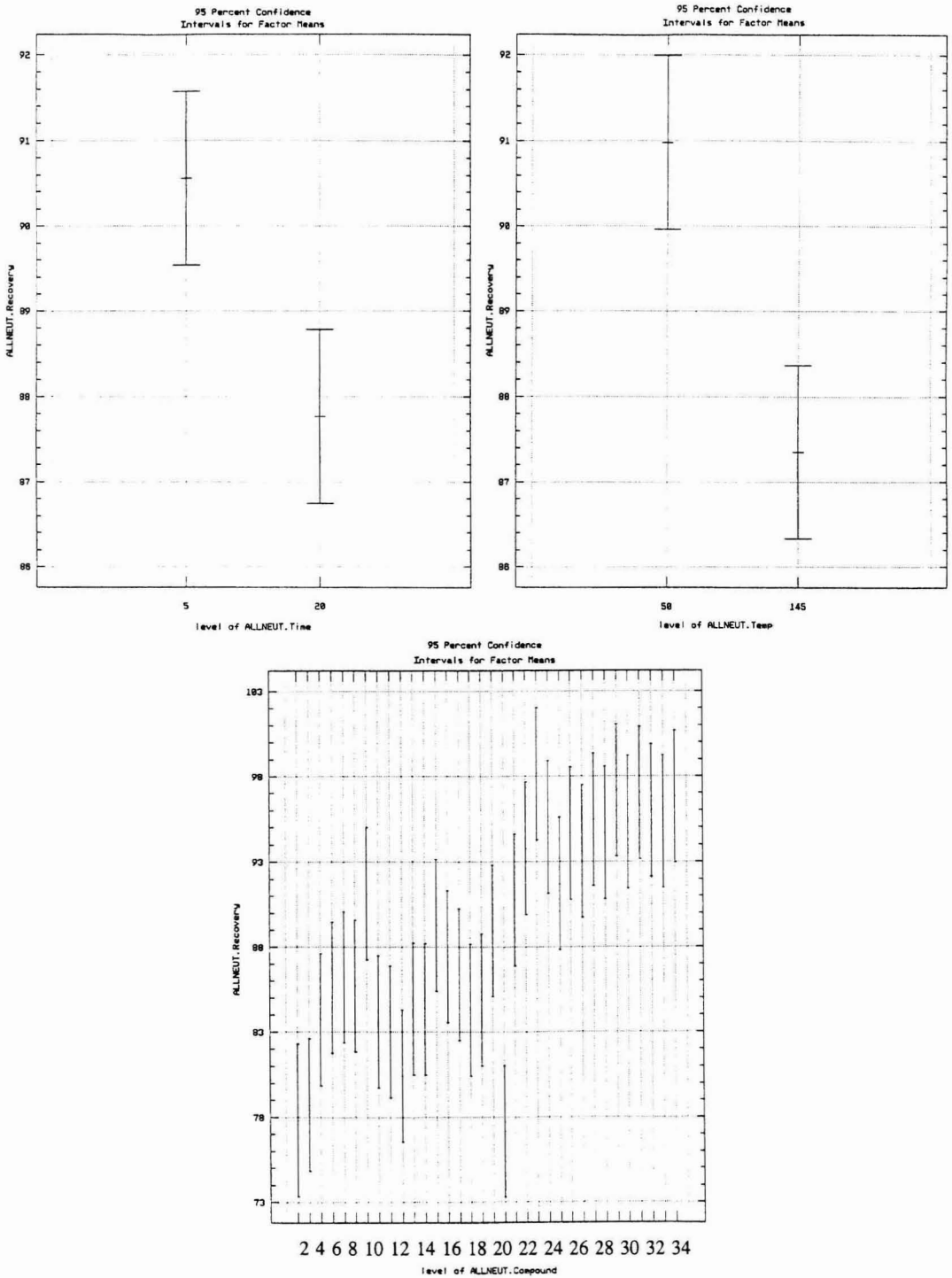

Figure 2. Recovery as a function of time, temperature, and compound for 34 neutral compounds: means and 95% confidence intervals for factor means. Compounds are arranged in order of elution from the GC column. For compound number, refer to Table 1.

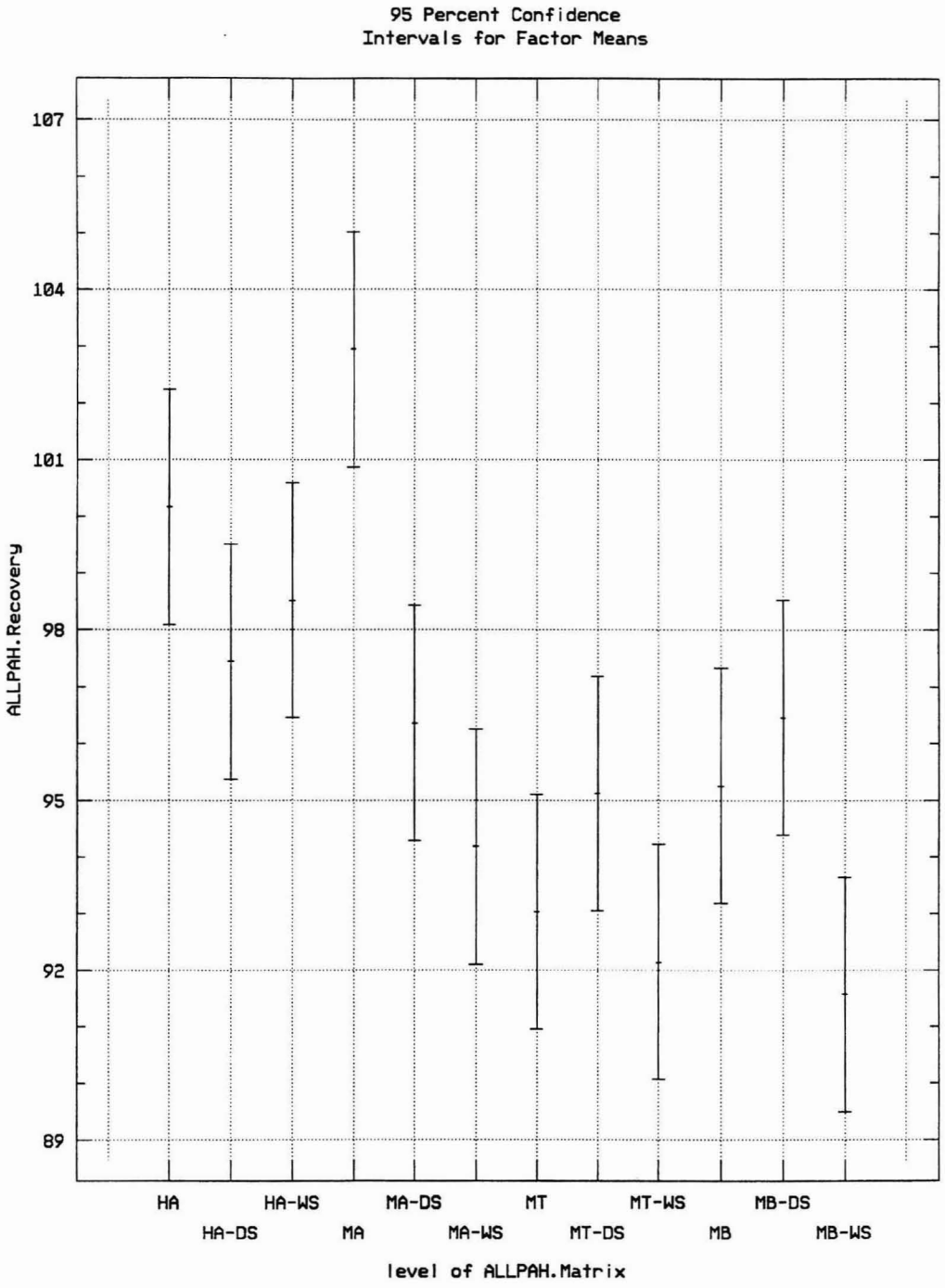

Figure 3. Recovery as a function of matrix for 22 PAHs: means and 95% confidence intervals for factor means.

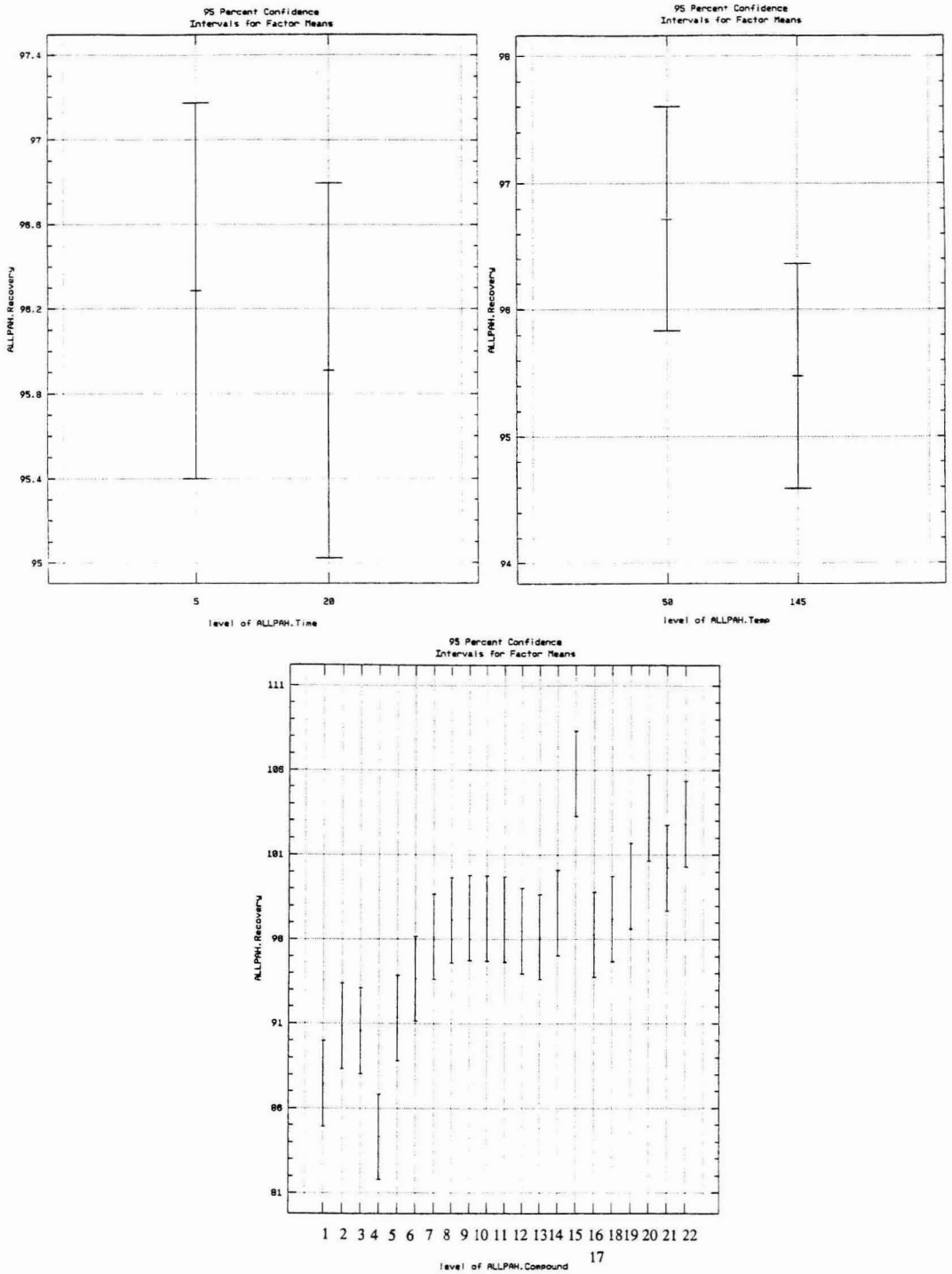

Figure 4. Recovery as a function of time, temperature, and compound for 22 PAHs: means and 95% confidence intervals for factor means. Compounds are arranged in order of elution from the GC column. For compound number, refer to Table 1.

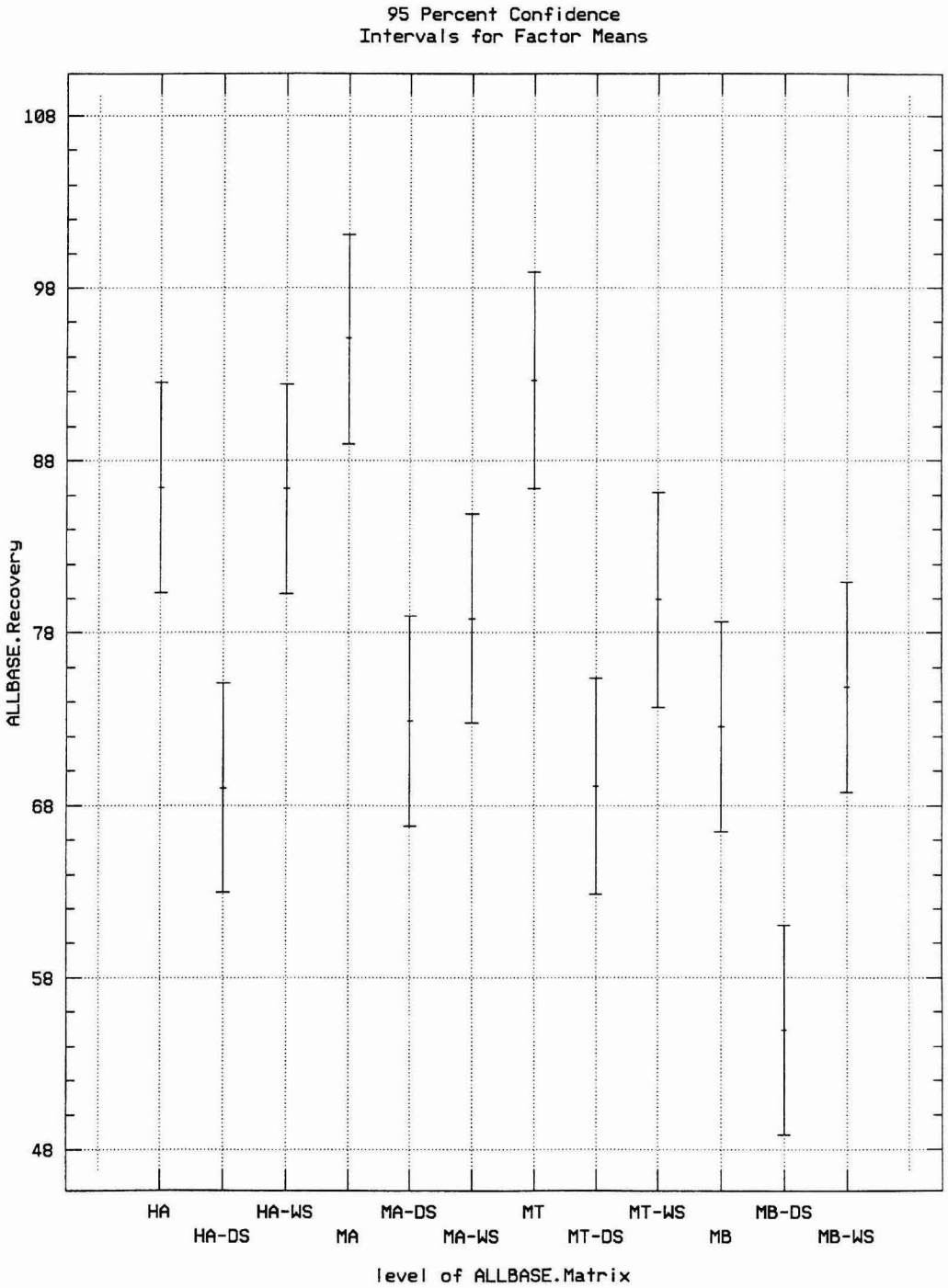

Figure 5. Recovery as a function of matrix for 19 basic compounds: means and 95% confidence intervals for factor means.

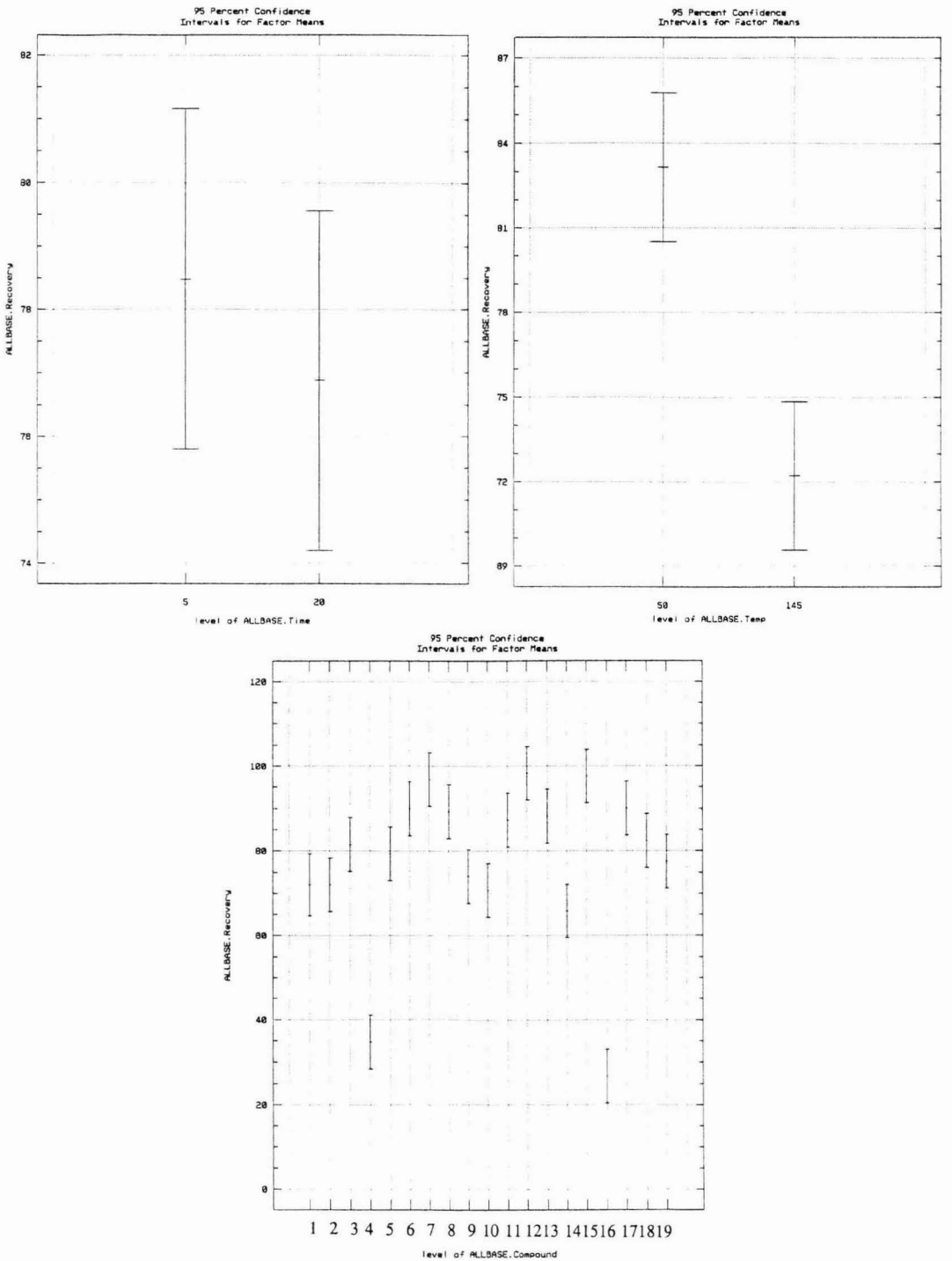

Figure 6. Recovery as a function of time, temperature, and compound for 19 basic compounds: means and 95% confidence intervals for factor means. Compounds are arranged in order of elution from the GC column. For compound number, refer to Table 1.

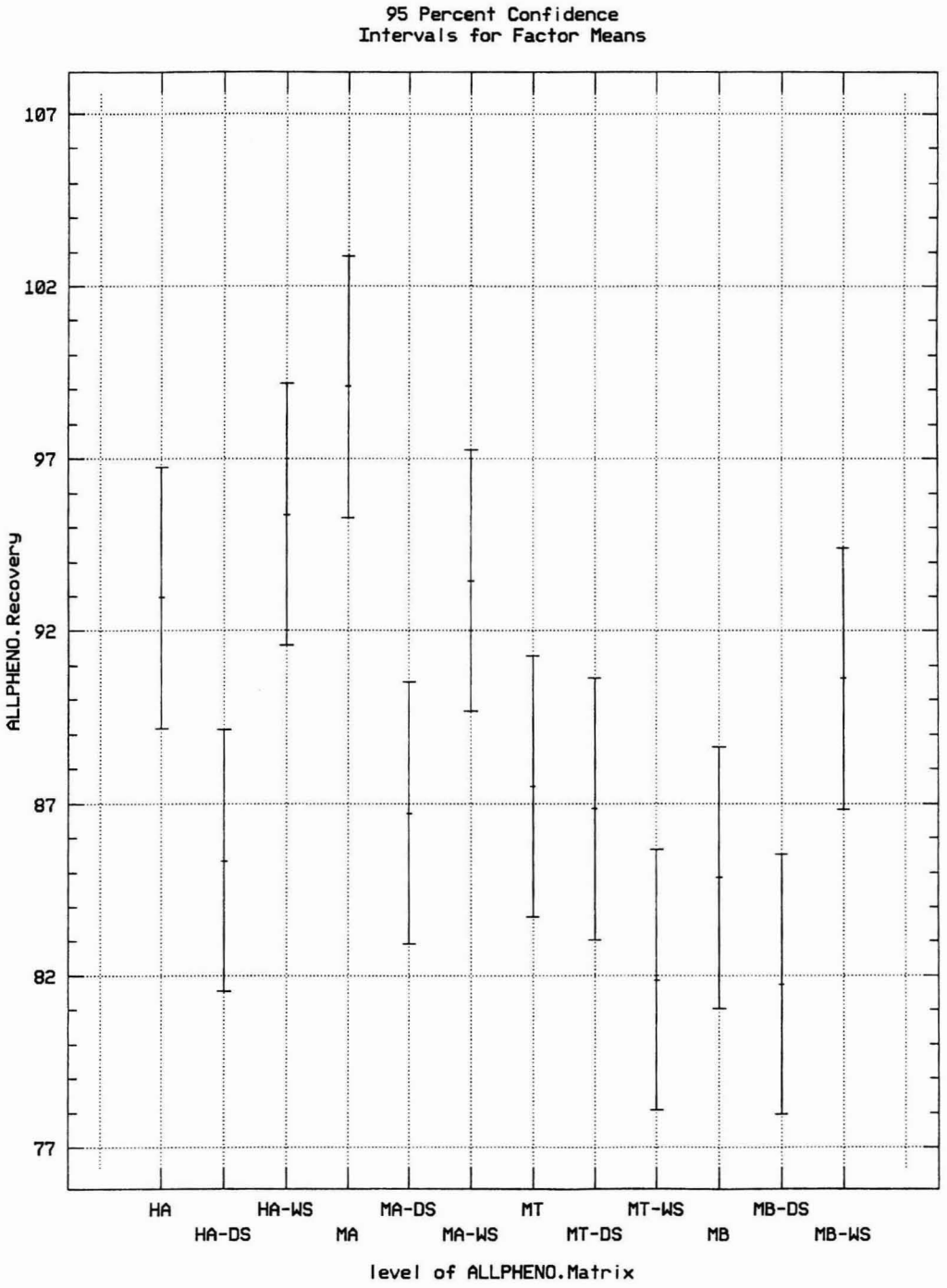

Figure 7. Recovery as a function of matrix for 19 phenolic compounds: means and 95% confidence intervals for factor means.

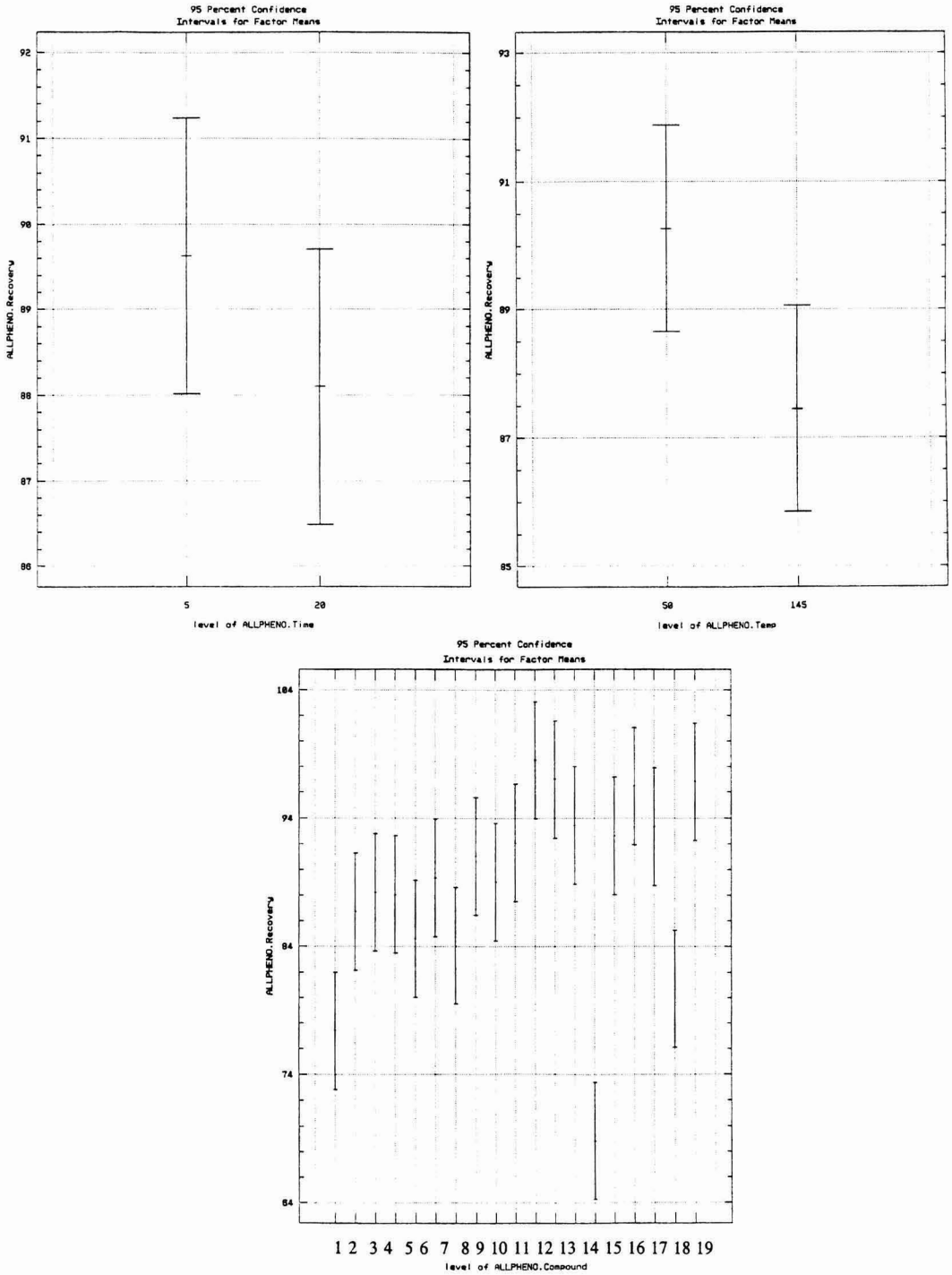

Figure 8. Recovery as a function of time, temperature, and compound for the 19 phenolic compounds: means and 95% confidence intervals for factor means. Compounds are arranged in order of elution from the GC column. For compound number, refer to Table 1.

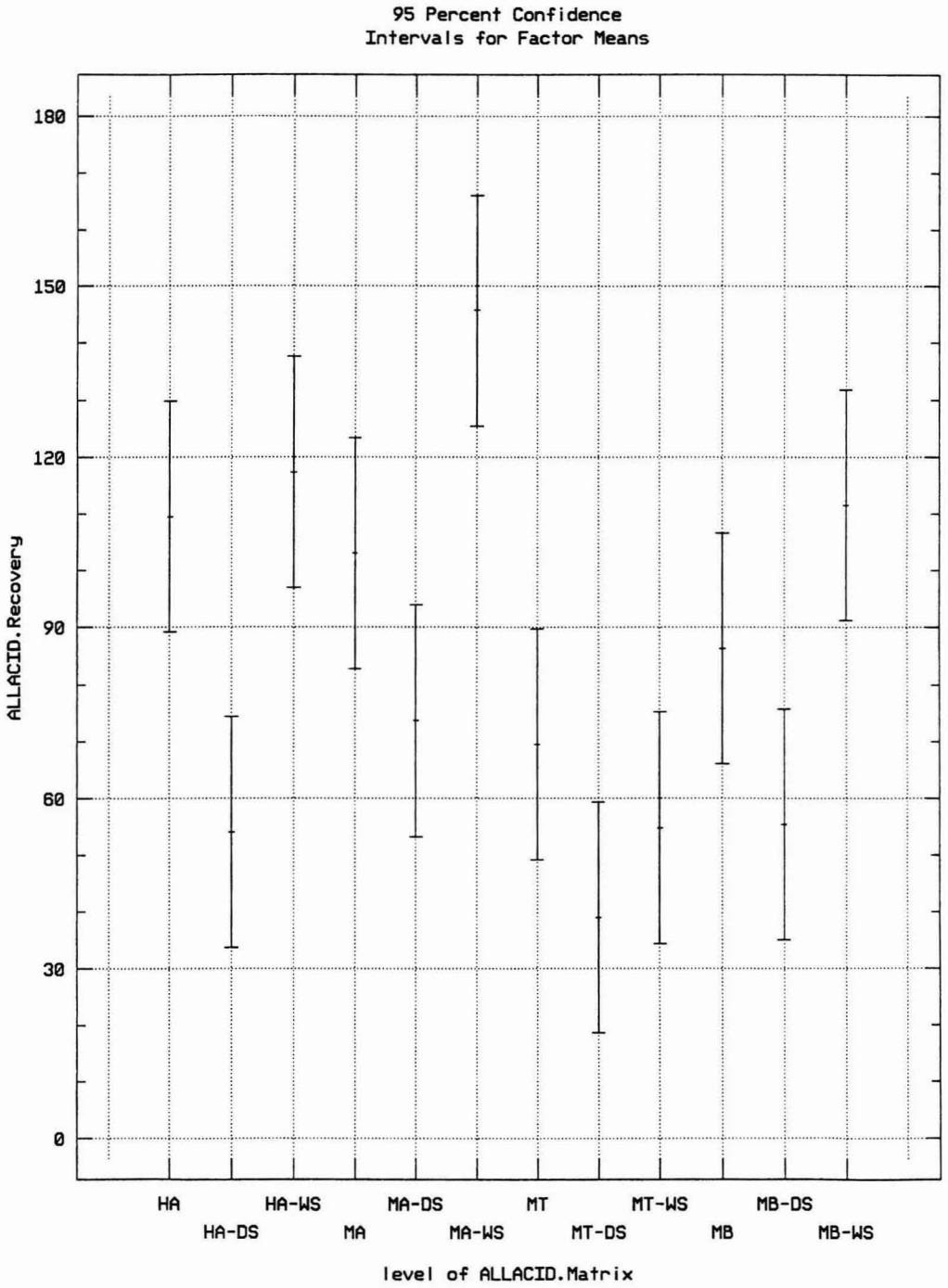

Figure 9. Recovery as a function of matrix for benzoic acid: means and 95% confidence intervals for factor means.

recovery was 88% when heated at 50°C, and only 30% when heated at 145°C in MA alone. When dry or wet soil was present, benzidine recoveries (for the 5 min time) dropped from 21% at 50°C to 1% at 145°C. This behavior of benzidine is not surprising; catalytic reactions in the presence of soil may have contributed to these low recoveries.

Of the 19 basic compounds, 11 had mean recoveries >80%. Recoveries were higher from the solvent/wet soil suspensions than from the solvent/dry soil suspensions.

Phenolic Compounds

Figures 7 and 8 summarize recovery data for 19 phenolic compounds. Mean recoveries were >80% for all solvents and solvent/soil suspension combinations, and neither time nor temperature seemed to have a significant effect on recovery. Except for MT, other solvent combinations seemed to give recoveries that were 6–10% higher when water was present in the soil matrix than when dry soil suspensions were used. 2-Fluorophenol, 2,4-dinitrophenol, and 4,6-dinitro-2-methylphenol gave lower recoveries. We have reported previously (2) that dinitrophenols gave lower recoveries when MAE was used, possibly because of catalytic reactions with soil components.

Benzoil Acid

The recovery data for benzoic acid are presented in Figures 9 and 10. There is significantly more spread in the recovery data for benzoic acid (Figure 9) than for other compounds, although neither temperature nor heating time appeared to have significant effect on recovery (Figure 10). The wide confidence intervals are a function of the small sample size in each case, because this group includes only one compound.

Conclusions

Overall, the solvent combination HA (1 + 1) seems to be the best for the compounds and matrixes investigated, with recoveries >80%, except for basic compounds and benzoic acid in the solvent/dry soil suspension experiments. Increasing the extraction time from 5 to 20 min did not increase recoveries; in fact, recoveries of neutral compounds decreased slightly at the longer extraction time. Increasing the temperature from

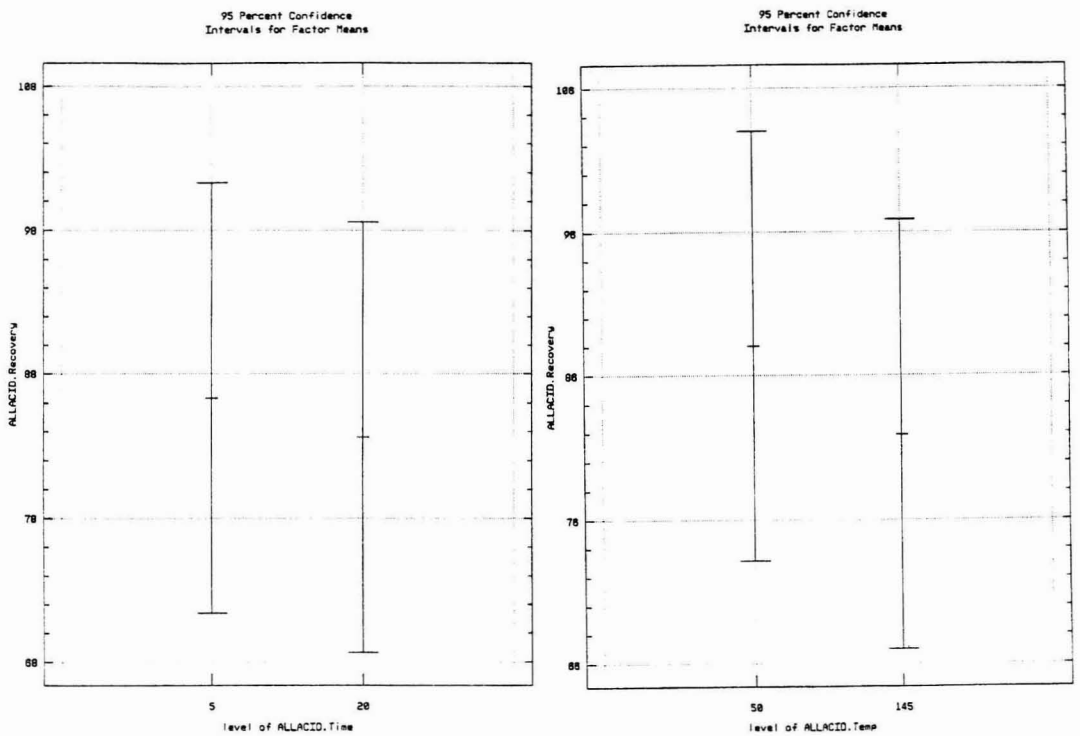

Figure 10. Recovery as a function of time and temperature for benzoic acid: means and 95% confidence intervals for factor means.

50° to 145°C decreased recoveries of basic compounds by about 10%. Recoveries of basic compounds, of benzoic acid (and probably other organic acids as well), and at least to some extent of phenolic compounds from the solvent/wet soil suspensions are higher than those for solvent/dry soil suspensions. The reason may be that, in solvent/wet soil suspensions, polar sites in the (formerly dry) soil are already occupied by water molecules before other polar molecules have a chance to compete for the site. However, the data are from freshly spiked materials. At present, it is not known to what extent these results could be duplicated with real-world samples.

Notice

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development (ORD), partially funded and collaborated in the research described here. This article has not been subjected to the Agency's review and has not been approved as an EPA publication. Neither the EPA nor the ORD endorses or recommends any trade names or commercial products mentioned in this article; they are noted solely for the purpose of description and clarification. The U.S. Government has a nonexclusive royalty-free license in and to any copyright covering this article.

Acknowledgments

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TECHNICAL COMMUNICATIONS

Ivermectin Quantitation with an Abamectin Internal Standard

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The potent antiparasitic ivermectin is detected at low concentrations (ng/g) in liver and muscle tissue by liquid chromatography after conversion to a fluorescent derivative with 1-methylimidazole and trifluoroacetic anhydride. This acetylation reaction can be compromised by residual water that leads to decreased yields. Yields of derivatives of ivermectin and abamectin, a related avermectin, are identical under all circumstances tested. Use of abamectin as an internal standard eliminates derivative yield as a source of analytical variation.

The presence of the antiparasitic animal drug ivermectin is monitored by the Food Safety and Inspection Service, an agency of the United States Department of Agriculture (1). Tissues analyzed for this compound are liver and muscle. The current ivermectin method is based on liquid chromatographic (LC) analysis of a fluorescent derivative of ivermectin (2). The sensitivity of the derivatization reaction to water affects conversion (3).

Abamectin, an avermectin that is structurally similar to ivermectin, is used primarily as an agricultural pesticide and is suitable for use as an internal standard (4, 5). The experiment and statistical analyses described here demonstrate the equivalent derivative conversion of both compounds within a series of standards and the variability of the conversion between these standards. Results of actual recoveries were calculated using ivermectin and ivermectin with abamectin as internal standards to demonstrate the compensating effects the use of abamectin as an internal standard has on quantitation of ivermectin.

Experimental

A set of 10 standards is prepared, each containing 75 ng abamectin (ABA) and 75 ng ivermectin (IVR). Solutions are reduced to dryness, the avermectins are derivatized, and they are determined by LC. The experiment is repeated on different days for a total of 3 sets.

The derivatization reaction is an acetylation using 1-methylimidazole and trifluoroacetic anhydride. The chromatography uses reversed-phase C_{18}, a mobile phase of methanol–water (97 + 3, v/v) at a flow rate of 1.8 mL/min, and fluorescence detection (375 nm for excitation and 470 nm for emission).

Each day's experiment provides 2 data groups (one each for ABA and IVR) representing analytical responses for spiked compounds. Because the analytical responses of ABA and IVR differ, results for each compound are normalized as a percentage of its mean values. A third data group is calculated by dividing the analytical response of IVR by the analytical response of ABA and normalizing in a similar fashion. The normalized data points of the 3 groups (ABA, IVR, and IVR/ABA) are used to calculate descriptive statistics. The absolute difference between each point and its respective mean for all of the groups is calculated. These results are used for analysis of inferential statistics.

The results of each set are analyzed statistically. Analysis of variance (ANOVA) is used to determine significant differences between ABA, IVR, and IVR/ABA data groups. Tukey's test for honest significant difference is used to determine which group or groups are significantly different.

The null hypothesis for the ANOVA tests is ABA = IVR = IVR/ABA. The alternative hypothesis is ABA IVR IVR/ABA. The test statistic is the F ratio at a one-tailed significance level of $\alpha = 0.05\%$. The rejection region is $F \geq 3.35$. The test statistic for Tukey's test is HSD = q_α where MSE (mean square for error) is calculated in the ANOVA test and $N = 10$. q_α is found in the Table for Percentages of the Studentized range with df = 27, $k = 3$, and $\alpha = 0.05\%$.

Actual analytical results are quantitated with external standard curves. An equal amount of ABA is added to standards and samples. A best-fit line is constructed from standards with IVR concentration as the dependent variable. The independent variables for IVR and IVR/ABA best-fit lines are the analytical response of

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Table 1. Analysis of variance data[a]

ANOVA Test of abamectin calculation study

Set 1

Sample	ABA	NORM ABA	X_i-MEAN ABS	$DIFF^2$	IVR	NORM IVR	X_i-MEAN ABS	DIFF	IVR/ABA	NORM IVR/ABA	X_i-MEAN ABS	$DIFF^2$
1	1163	94.62	5.38	28.92	807	96.35	3.65	13.35	0.69	101.81	1.81	3.29
2	1145	93.16	6.84	46.82	772	92.17	7.83	61.34	0.67	98.93	1.07	1.15
3	1279	104.06	4.06	16.48	870	103.87	3.87	14.96	0.68	99.81	0.19	0.04
4	1257	102.27	2.27	5.15	855	102.08	2.08	4.32	0.68	99.80	0.20	0.04
5	1059	86.16	13.84	191.53	728	86.91	13.09	171.22	0.69	100.87	0.87	0.75
6	1193	97.06	2.94	8.63	804	95.99	4.01	16.09	0.67	98.88	1.12	1.25
7	1263	102.76	2.76	7.61	867	103.51	3.51	12.32	0.69	100.72	0.72	0.52
8	1331	108.29	8.29	68.72	916	109.36	9.36	87.61	0.69	100.98	0.98	0.96
9	1321	107.48	7.48	55.91	901	107.57	7.57	57.29	0.68	100.08	0.08	0.01
10	1280	104.14	4.14	17.15	856	102.20	2.20	4.83	0.67	98.12	1.88	3.52
MEAN	1229	100.00	5.80		838	100.00	5.72		0.68	100.00	0.89	
SUM X			57.99				57.16				8.91	
SUM X^2				446.93				443.32				11.51
SS	110.60				116.56				3.57			
SS_{tot}	388.67											
SS_w	230.73		DF_w =		27	MSE =		8.55		F =	9.24	
SS_{bet}	157.93		DF_{bet} =		2	MSE =		78.97		REJECTION REGION >= 3.35		

Tukey's honest significant difference test

Difference between means matrix

		Mean 1	Mean 2	Mean 3
		5.80	5.72	0.89
Mean 1	5.80		0.08	4.91
Mean 2	5.72			4.83
Mean 3	0.89			
Mean differences are significantly ≥			3.24	

Set 2

Sample	ABA	NORM ABA	X_i-MEAN ABS	$DIFF^2$	IVR	NORM IVR	X_i-MEAN ABS	$DIFF^2$	IVR/ABA	NORM IVR/ABA	X_i-MEAN ABS	$DIFF^2$
11	1457	111.88	11.88	141.11	1037	110.28	10.28	105.76	0.71	98.58	1.42	2.01
12	1263	96.98	3.02	9.11	880	93.59	6.41	41.12	0.70	96.51	3.49	12.20
13	1239	95.14	4.86	23.63	910	96.78	3.22	10.38	0.73	101.73	1.73	3.00
14	1353	103.89	3.89	15.16	972	103.37	3.37	11.37	0.72	99.51	0.49	0.24
15	1309	100.51	0.51	0.26	937	99.65	0.35	0.12	0.72	99.15	0.85	0.73
16	1200	92.14	7.86	61.71	859	91.35	8.65	74.76	0.72	99.15	0.85	0.72
17	1227	94.22	5.78	33.43	885	94.12	5.88	34.59	0.72	99.90	0.10	0.01
18	1421	109.11	9.11	83.08	1065	113.26	13.26	175.87	0.75	103.81	3.81	14.52
19	1317	101.13	1.13	1.27	939	99.86	0.14	0.02	0.71	98.76	1.24	1.55
20	1237	94.99	5.01	25.14	919	97.73	2.27	5.13	0.74	102.90	2.90	8.43
MEAN	1302	100.00	5.31		940	100.00	5.38		0.72	100.00	1.69	
SUM X			53.06				53.83				16.89	
SUM S^2				393.90				459.12				43.39
SS	112.36				169.32				14.87			
SS_{tot}	385.66											
SS_w	296.54		DF_w =		27	MSE =		10.98		F =	4.06	
SS_{bet}	89.13		DF_{bet}		2	MSE =		44.57		REJECTION REGION >= 3.35		

Difference between means matrix

		Mean 1	Mean 2	Mean 3
		5.31	5.38	1.69
Mean 1	5.31		0.07	3.62
Mean 2	5.38			3.69
Mean 3	1.69			
Mean differences are significantly ≥			3.68	

Table 1. (*continued*)

ANOVA Test of abamectin calculation study — Set 3

Sample	ABA	NORM ABA	X_i-MEAN ABS	$DIFF^2$	IVR	NORM IVR	X_i-MEAN ABS	$DIFF^2$	IVR/ABA	NORM IVR/ABA	X_i-MEAN ABS	$DIFF^2$
21	1372	102.45	2.45	6.00	980	104.43	4.43	19.65	0.71	101.97	1.97	3.88
22	1247	93.12	6.88	47.40	848	90.37	9.63	92.80	0.68	97.08	2.92	8.53
23	1343	100.28	0.28	0.08	934	99.53	0.47	0.22	0.70	99.28	0.72	0.52
24	1285	95..95	4.05	16.38	890	94.84	5.16	26.60	0.69	98.87	1.13	1.27
25	1293	96.55	3.45	11.90	907	96.65	3.35	11.20	0.70	100.14	0.14	0.02
26	1329	99.24	0.76	0.58	937	99.85	0.15	0.02	0.71	100.65	0.65	0.42
27	1289	96.25	3.75	14.05	911	97.08	2.92	8.53	0.71	100.89	0.89	0.80
28	1371	102.37	2.37	5.64	960	102.30	2.30	5.30	0.70	99.96	0.04	0.00
29	1449	108.20	8.20	67.22	1020	108.70	8.70	75.61	0.70	100.49	0.49	0.24
30	1414	105.59	5.59	31.20	997	106.24	6.24	39.00	0.71	100.66	0.66	0.43
MEAN	1339	100.00	3.78		938	100.00	4.34		0.70	100.00	0.96	
SUM X			37.78				43.35				9.60	
SUM X^2				200.45				278.93				16.11
SS	57.69				91.00				6.88			
SS_{tot}	221.03											
SS_w	155.57		DF_w =		27	MSE =		5.76		F =	5.68	
SS_{bet}	65.46		DF_{bet} =		2	MSE =		32.73		REJECTION REGION >= 3.35		

Tukey's honest significant difference test — Difference between means matrix

		Mean 1	Mean 2	Mean 3
		3.78	4.34	0.96
Mean 1	3.78		0.56	2.82
Mean 2	4.34			3.38
Mean 3	0.96			
Mean differences are significantly ≥			2.66	

[a] ABA = abamectin peak height; IVR = ivermectin peak height; IVR/ABA = ivermectin peak height divided by abamectin peak height; NORM ABA = normalized ABA value; NORM IVR = normalized IVR value; NORM IVR/ABA = normalized IVR/ABA value; X_i-MEAN ABS = absolute difference between the normalized value and its mean; $DIFF^2$ = the square of X_i-MEAN ABS; SS = sum of square; SS_{tot} = sum of square within; SS_{bet} = sum of square between; DF_w = degrees of freedom within; DF_{bet} = degrees of freedom between; and MSE = mean square for error.

the IVR and the IVR/ABA ratio, respectively. The equation $Y = MX + B$ is used to calculate IVR concentrations in samples. In this equation, Y = sample concentration, M = slope of best-fit line, B = Y intercept of best-fit line, and X sample's analytical response (IVR or IVR/ABA).

Results

Results for each set's statistical analysis are found in Table 1. The table contains analytical responses recorded from the LC detector for each compound, normalized responses, renormalized data, ANOVA, and Tukey's tests of the 3 sets.

Each of the 3 ANOVA tests produced F ratios in the rejection region, leading to rejection of the null hypothesis. IVR means are significantly higher than IVR/ABA means in all 3 HSD tests. The difference between ABA and IVR/ABA means are significantly higher in the HSD tests of sets 1 and 3. No significant differences were found between means of ABA and IVR in any of the 3 sets.

Figure 1 shows the dispersion of the compounds around their means. The graph demonstrates the similarity between the normalized values of each compound in individual standards and the ranges for normalized values. The largest difference between the compound's normalized values in any of the 30 standards is 4.15% (Table 1, set 2, standard 18), while the ranges are in excess of 18% in each of the 3 sets.

It describes the distribution between the compounds and their ratios in the combined sets. Thirteen of the standards have both compounds above their means, 14 standards have both compounds below their means, and 3 standards have ABA values above and IVR below their means. Sixteen of the standards have higher ABA values than IVR values, and 14 standards have IVR higher values.

Individual compounds are evenly distributed around the common mean. Twenty-nine of the 60 values are above the mean and 31 are below. Sixteen of the 29 values above the mean are ABA and 13 are IVR. Fourteen of the values below the mean are ABA and 17 are IVR. The derivative conversion of the 2 compounds is equivalent.

Discussion

Higher normalized values for the spiked compounds represent a more complete conversion during derivatization, and lower values, a less complete conversion. The distributions of the spiked compounds demonstrate the variability of the derivative conversion. The values of the spiked compounds depend on the quality of the conversion. Because the compounds convert equivalently regardless of the derivatization's completeness, using ABA as an internal standard eliminates derivative conversion as a source of variation. Adding a known amount of ABA to each sample allows quantitation with the IVR/ABA ratio by comparing it to external standard ratios of known concentrations.

The 3 sets of calculation comparison data (Table 2) illustrate these conclusions. The sets represent analyst familiarization data calculated against standard curves using both IVR and IVR/ABA responses. Within-set differences between the 2 techniques' descriptive statistics demonstrate the utility of the ABA internal standard. Differences between the sets can be attributed to the analyst's technique.

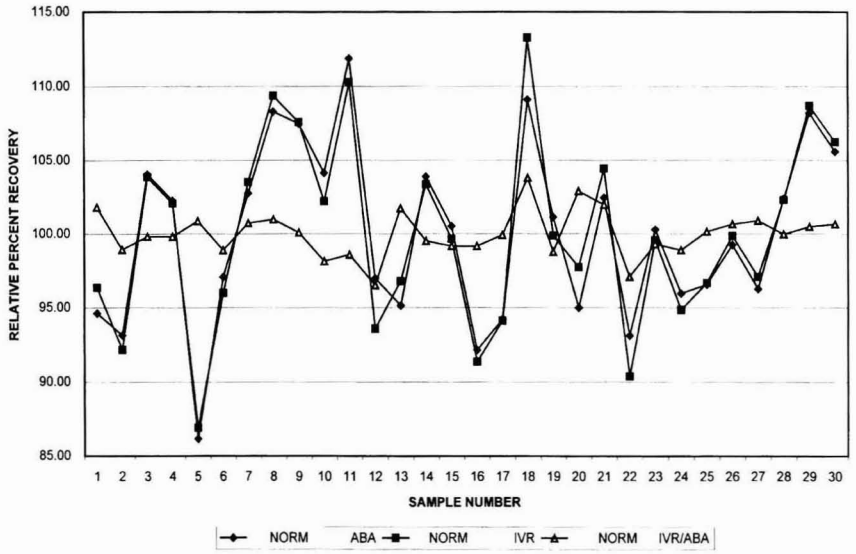

Figure 1. Relative compound recovery.

Table 2. Calculation comparison study[a]

Sample No.	Spike AMT	Peak height ABA	Peak height IVR	IVR ABA	ISTD corrected results: IVR ABA, ppb	ISTD corrected results: REC IVR ABA, %	Uncorrected results: IVR, ppb	Uncorrected results: REC IVR, %
			Set 1					
1	10	1899	549	0.29	10.35	103.48	9.04	90.39
2	60	1732	3047	1.76	61.31	102.18	53.19	88.65
3	20	1781	1023	0.57	20.24	101.19	17.42	87.08
4	15.0	1781	757	0.43	15.06	100.40	12.72	84.77
5	45.0	1688	2209	1.31	45.69	101.53	38.38	85.29
6	45	1549	1931	1.25	43.54	96.76	33.47	74.37
7	7.5	1517	291	0.19	6.98	93.01	4.48	59.72
8	40	1701	1893	1.11	38.90	97.26	32.79	81.98
9	20	1724	993	0.58	20.29	101.46	16.89	84.43
10	10	1788	525	0.29	10.50	105.05	8.62	86.15
11	15	1485	620	0.42	14.80	98.66	10.29	68.63
12	30	1475	1259	0.85	29.92	99.72	21.59	71.96
13	55	1240	2096	1.69	58.92	107.13	36.38	66.15
			Set 2					
14	60	707	1513	2.14	55.95	93.26	46.40	77.33
15	15	741	371	0.50	13.37	89.15	11.38	75.87
16	45	795	1176	1.48	38.79	86.20	36.07	80.15
17	38	523	719	1.37	36.08	96.20	22.05	58.81
18	8	653	173	0.26	7.25	96.67	5.31	70.80
19	20	501	353	0.70	18.67	93.35	10.83	54.15
20	40	597	821	1.38	36.09	90.22	25.18	62.95
21	15	585	317	0.54	14.44	96.29	9.73	64.84
22	10	435	149	0.34	9.27	92.65	4.57	45.74
23	30	503	511	1.02	26.76	89.19	15.67	52.25
24	10	665	220	0.33	8.96	89.62	6.75	67.51
25	45	645	1021	1.58	41.48	92.19	31.31	69.58

Standard curve

Std. ppb	ABA	IVR	IVR ABA
0	2166	0	0.00
7.5	2240	472	0.21
15	2224	915	0.41
30	2063	1751	0.85
60	1977	3415	1.73

Regression output: IVR ABA

Slope	34.66497
Y Intercept	0.32646
r Squared	0.99991

Regression output: IVR

Slope	0.01767
Y Intercept	−0.66390
r Squared	0.99980

Standard curve

Std Ppb	AB	IVR	IVR ABA
0	820	0	0.00
8	820	223	0.27
15	887	502	0.57
30	895	997	1.11
60	843	1946	2.31

Regression output: IVR/ABA

Slope	25.97399
Y intercept	0.36864
r squared	0.99984

Regression output: IVR

Slope	0.03066
Y intercept	0.00491
r squared	0.99977

Table 2. (*continued*)

Sample No.	Spike AMT	Peak height ABA	Peak height IVR	IVR/ABA	ISTD corrected results: IVR/ABA, ppb	ISTD corrected results: REC IVR/ABA, %	Uncorrected results: IVR, ppb	Uncorrected results: REC IVR, %
			Set 3					
26	60	1064	1856	1.74	61.39	102.31	67.64	112.73
27	15	993	442	0.45	15.59	103.90	15.49	103.29
28	45	821	1065	1.30	45.62	101.38	38.47	85.48
29	30.0	979	864	0.88	31.01	103.35	31.06	103.52
30	7.5	827	179	0.22	7.52	100.33	5.80	77.27
31	20	916	517	0.56	19.79	98.95	18.26	91.30
32	40	808	928	1.15	40.38	100.95	33.42	83.54
33	15	808	332	0.41	14.38	95.86	11.44	76.25
34	55	949	1395	1.47	51.71	94.02	56.64	92.07
35	10	852	259	0.30	10.61	106.11	8.75	87.45
36	30	893	823	0.92	32.38	107.94	29.54	98.48
37	20	632	398	0.63	22.09	110.47	13.87	69.36

Standard curve

Std, ppb	ABA	IVR	IVR/ABA
0	1040	0	0.00
7.5	995	212	0.21
15	1011	439	0.43
30	1043	893	0.86
60	949	1616	1.70

Regression output: IVR/ABA

Slope	35.25128
Y intercept	−0.10536
r squared	0.99998

Regression output: IVR

Slope	0.03688
Y intercept	−0.80559
r squared	0.99844

		ISTD corrected results	Uncorrected results	
Set 1	Mean	100.60	Mean	79.20
	Std	3.69	Std	9.86
	Cov	3.67	Cov	12.44
Set 2	Mean	92.08	Mean	65.00
	Std	3.16	Std	10.23
	Cov	3.44	Cov	15.73
Set 3	Mean	102.13	Mean	90.06
	Std	4.71	Std	12.79
	Cov	4.61	Cov	14.20

[a] ISTD = internal standard; STD = standard deviation; and COV = coefficient of variation.

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TECHNICAL COMMUNICATIONS

Solid-Phase Extraction Cleanup for Ivermectin in Liver Tissue

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Extraction of liver tissue with organic solvent produces coextractants with compounds of interest. The solid-phase extraction (SPE) cleanup of liver tissue developed for ivermectin removes nonpolar coextractants. Liver extract that has been reduced to dryness is reconstituted in 0.5 mL acetonitrile. The mixture is passed through 0.1 g C_{18} SPE column, and the eluate is collected. The column is eluted further with 2 mL acetonitrile. Combined eluates are derivatized with 1-methylimidazole and trifluoroacetic anhydride, and the ivermectin derivative is determined by liquid chromatography with fluorescence detection.

Ivermectin is a widely used potent antiparasitic animal drug. The U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) monitors the nation's meat supply for the presence of this drug. Currently, the agency tests for the presence of ivermectin in 6800 samples/year.

Extracts of liver tissue often contain coextracted constituents that interfere with quantitation (1, 2). In the method used by FSIS to analyze liver and muscle tissue for ivermectin, acetonitrile extraction of a 2.5 g sample of liver tissue produces a group of nonpolar coextractants (3). This paper describes a solid-phase extraction (SPE) technique that removes coextracted constituents prior to the determinative step. The technique is compatible with any extraction procedure that produces interfering coextractants that are less polar than ivermectin.

METHOD

Apparatus

(a) *SPE column.*—C_{18}, 0.1 g, PN WAT023590 (Millipore Corp., Waters Chromatographic Div., Milford, MA), or equivalent.

(b) *Analytical column.*—Zorbax ODS 150 × 4.6 mm id (DuPont Co., Wilmington, DE), or equivalent.

Reagents

(a) *1-Methylimidazole.*—PN 33,609-2 (Aldrich Chemical, Milwaukee, WI), or equivalent.

(b) *Trifluoroacetic anhydride.*—PN 10623-2 (Aldrich), or equivalent.

(c) *Acetonitrile.*—Catalogue No. 015-4 (Baxter Healthcare Corp., Burdick & Jackson Div., Muskegon, MI), or equivalent.

Standards

(a) *Abamectin.*—Catalogue No. L$676-863-038A003 (Merck Sharpe & Dohme, Rahway, NJ), 500 ng/mL in acetonitrile.

(b) *Ivermectin.*—Catalogue No. L$640-471-076P004 (Merck Sharpe & Dohme), 500 ng/mL in acetonitrile.

Analysis

Determine elution pattern of ivermectin and abamectin on SPE column by adding 0.5 mL acetonitrile containing 150 ng each compound onto the column and eluting with 6 mL acetonitrile. Collect 0.5 mL fractions from column, derivatize fraction, and analyze by liquid chromatography (LC) to determine analyte retention volumes.

Verify SPE column's ability to retain coextractants by running a matched pair of samples of liver tissue known to contain coextracting material. Spike both samples with 150 ng abamectin and 75 ng ivermectin. Run one sample without SPE step and the other with the SPE step. Use an extended 70 min run time for both samples.

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Test performance of SPE cleanup parameters by running 3 sets of 15 randomly selected liver samples. Use abamectin as an internal standard, and spike samples with ivermectin at various levels. The first set consists of 3 recovery curves spiked with ivermectin by the analyst at 7.5, 15, 30, 45, and 60 ppb. Samples for the second and third sets are spiked blind to the analyst and contain a blank.

Completely evaporate acetonitrile used to extract compounds from liver tissue (3). Reconstitute dried liver extract with 0.5 mL acetonitrile. Prewet C_{18} SPE column with 1 mL acetonitrile. Discard the acetonitrile used to prewet column. Do not allow column to dry before adding sample. Transfer reconstituted sample onto column and collect eluate. Immediately elute column with a predetermined amount of acetonitrile (determined from fraction experiment) and combine eluates.

Prepare 3-point external standard curve. Make standards with same amount of abamectin added to samples and with ivermectin levels that approximate spiking levels. Evaporate solvent from standards prior to derivatization.

Derivatize samples by adding 0.2 mL 1-methylimidazole (1 + 1 v/v in acetonitrile) and 0.2 mL trifluoroacetic anhydride (1 + 1 v/v in acetonitrile) to combined eluates and shaking on Vortex mixer (4). Derivatize standards at same conditions and take them to a final volume of 2.5 mL with acetonitrile. Discard unused derivatizing reagent.

Analyze derivatives by LC using a C_{18} analytical column (4.5 mm × 15 cm containing 10 μm particle size) heated to 30° C, a mobile phase of methanol–water (97 + 3) at a flow rate of 1.8 mL/min, and fluorescence detection (excitation at 364 nm and emission at 480 nm). Run time is 15 min. Injection volume is 100 μL.

Quantitate by linear regression and best fit line constructed from 3-point external standard curve. Compute ratio of analytical response of ivermectin to that of abamectin for each standard. Use this ratio as the independent variable and the corresponding ivermectin concentration as the dependent variable. Calculate levels of ivermectin recovered from test samples using $y = mx + b$, where y is ivermectin concentration (ppb), x is ivermectin/abamectin ratio, m is slope, and b is y intercept.

Results and Discussion

Abamectin and ivermectin were fully eluted from the C_{18} column with 2 mL acetonitrile in gravity-

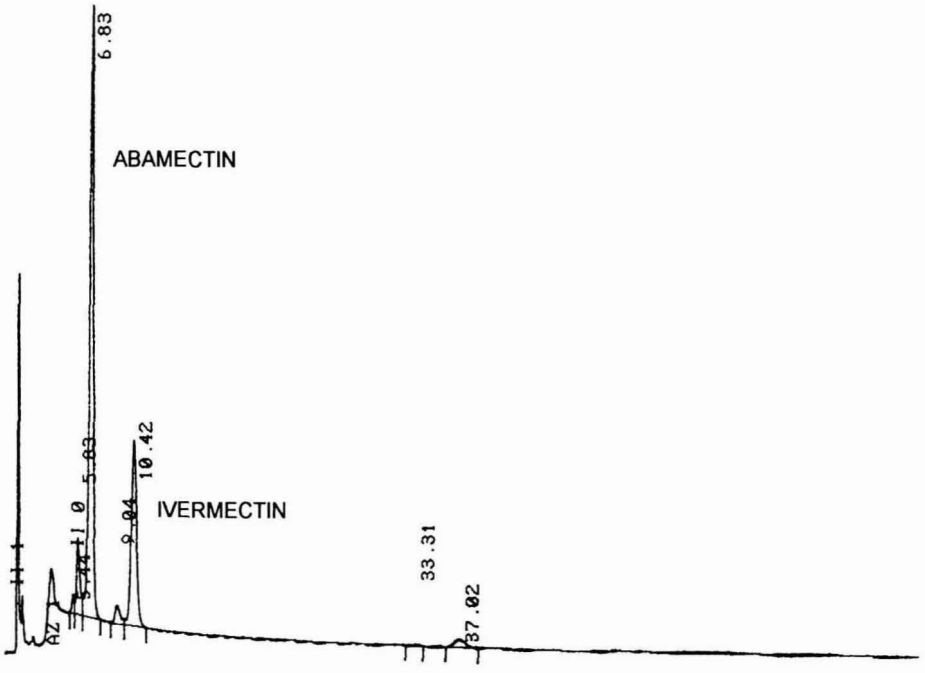

Figure 1. Liquid chromatogram of liver tissue obtained with C_{18} treatment.

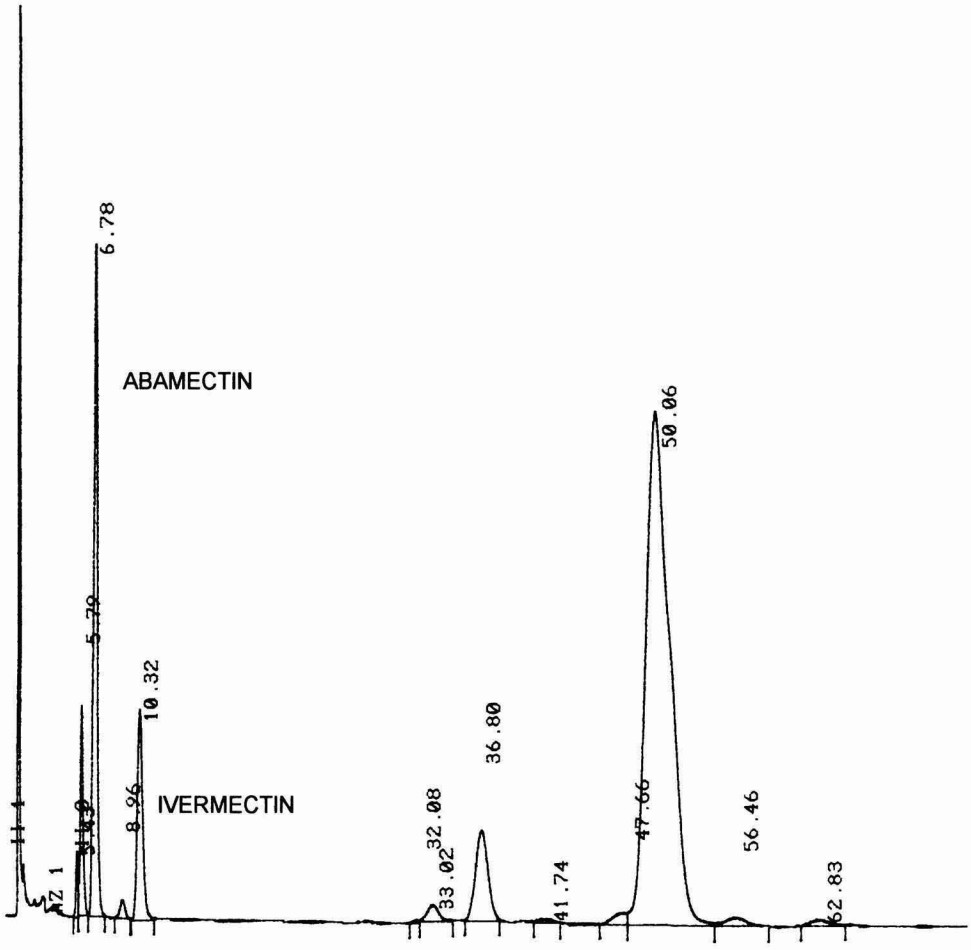

Figure 2. Liquid chromatogram of liver tissue obtained without C_{18} treatment.

induced flow. Chromatograms of liver tissue containing coextractants run with (Figure 1) and without (Figure 2) C_{18} treatment show that the column's loading capacity is adequate for the sample size.

Results of analyses of spiked samples are presented in Figure 3. Data from all 3 sets were combined, and results are presented in ascending order of ivermectin spike (7.5–63 ppm). The distribution of the recoveries is independent of the amount spiked.

Performance statistics are given in Table 1. Coefficients of variation were uniformly low for all 3 sets of data. Correlation coefficients calculated by using ivermectin/abamectin peak height ratios as the independent variable and the corresponding spiking levels as the dependent variable were uniformly high for each data set.

Previously, FSIS used column-switching valves to eliminate coextractants. The mechanical complexity of the equipment caused an unacceptable level of instrument downtime. An LC column gradient was adopted to flush the analytical column after each sample injection. This method proved more reliable, but it increased solvent use, decreased sample throughput because of extended LC run times, and further increased the complexity of the LC equipment. The SPE cleanup step described here is simple to use and requires no special equipment. Adoption of this step eliminates the need for gradient LC equipment and reduces analysis time without affecting quantitative performance.

Table 1. Performance statistics

Data set	Mean recovery, %	Standard deviation	Coefficient of variation	r	Standard error
Set 1	89.5	6.61	7.39	0.9950	1.95
Set 2	89.7	4.48	5.00	0.9988	0.95
Set 3	88.0	6.93	7.88	0.9965	1.73

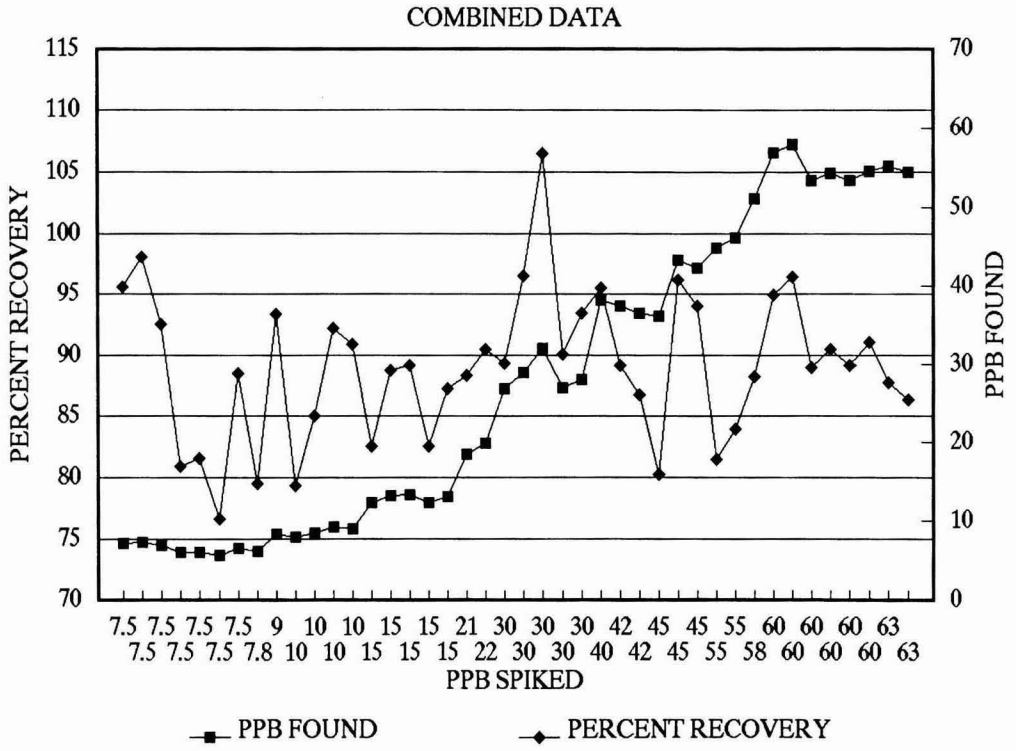

Figure 3. C_{18} SPE study combined data.

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TECHNICAL COMMUNICATIONS

Determination of 4-Hexylresorcinol in Crab Meat

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A method is described for determining 4-hexylresorcinol in crab meat. 4-Hexylresorcinol is used to prevent melanosis in shrimp, and the same use has been proposed for crab meat. Because 4-hexylresorcinol may be added illegally to crab meat as a preservative, consumer protection requires that residues of the compound be monitored in crab meat. 4-Hexylresorcinol is extracted from crab meat with acetonitrile. After dilution with water, the extract is passed through a C_{18} solid-phase extraction column and 4-hexylresorcinol is eluted from the column with ethanol. The compound is determined by reversed-phase liquid chromatography with diode array detection at 206 nm. Limit of quantitation is 1.0 μg/g. Mean recovery in the range 1–20 μg/g is 89%, with a relative standard deviation of 6.3.

Once a crustacean, such as a crab, is harvested, black spots referred to as melanosis start to form (1). Although the crab's meat is not spoiled, the commercial value of the crab is compromised because it appears unpalatable. Melanosis is caused by polyphenol oxidase, an enzyme present in many foods (1–6). Use of 4-hexylresorcinol to prevent melanosis has been suggested because it strongly inhibits polyphenol oxidase (2). In addition, 4-hexylresorcinol is economical and odorless, does not stain, and is relatively nontoxic (2, 7, 8). At present, 4-hexylresorcinol is used to prevent melanosis in shrimp (6, 8).

Although 4-hexylresorcinol has an evaluation of "Generally Recognized as Safe" (GRAS) for shrimp (8), no GRAS evaluation has been made for crab meat. If 4-hexylresorcinol is used in crab meat to prevent melanosis, then it would be considered a food additive and its levels should be monitored.

Some individuals are sensitive to 4-hexylresorcinol (7). Its use as an antiseptic and anthelmintic shows 4-hexylresorcinol can be toxic and irritating (9). For this reason, 4-hexylresorcinol is not used directly. Rather, it is dissolved in some solvent before use. For example, a 1:1000 dilution is a safe concentration for its use as a pharyngeal antiseptic (7).

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A method is needed to detect unauthorized use of 4-hexylresorcinol in crab meat. To date, only one procedure for measuring 4-hexylresorcinol in shrimp has been reported (6). The reported 4-hexylresorcinol residual level for treated shrimp is <1.0 ppm (6). No methods or residual levels have been reported for crab meat treated with 4-hexylresorcinol. The following method for crab meat was developed on the basis that treatment protocol and levels of 4-hexylresorcinol residues would be same as for shrimp. Analysis time is 4–5 min, and run is isocratic.

METHOD

Reagents

(a) *Liquid chromatographic (LC) grade acetonitrile, United States Pharmacopeia grade ethanol, deionized water.*

(b) *4-Hexylresorcinol.*—Sigma Chemical Co. (St. Louis, MO).

(c) *4-Hexylresorcinol stock standard.*—1 mg/mL in ethanol.

(d) *Saturated sodium chloride solution.*—35.7 g sodium chloride/100 mL deionized water.

(e) *Working standards.*—0.5–80 μg 4-hexylresorcinol/mL, appropriate dilutions of stock standard with ethanol.

(f) *Equilibrating solutions.*—Appropriate dilutions of 4-hexylresorcinol stock standard with saturated sodium chloride solution.

(g) *Solid-phase extraction (SPE) columns.*—C_{18}, 500 mg, Bond Elut (Varian, Harbor City, CA).

(h) *Mobile phase.*—Acetonitrile–deionized water (75 + 25).

(i) *Analytical column.*—C_{18} reversed-phase, base deactivated, 5 μm, 4.6 × 250 mm, Supelco pKb-100 or equivalent (Supelco, Bellefonte, PA).

Apparatus

The LC system consists of a Waters Model 600E solvent delivery system, Model 991 diode-array LC detector, and Model 715 sample processor controlled by an NEC-386 computer and with Waters 6A-6.22a diode array software (Waters, Millford, MA).

Preparation of Crab Meat Containing 4-Hexylresorcinol

Remove crab meat from crab claws and composite. Place ca 20, 16, and 10 g composited crab meat in separate beakers containing 50 mL saturated sodium chloride solution. Beginning with beaker containing 20 g, add respectively 20, 160, and 200 μL 4-hexylresorcinol stock solution (1 mg/mL). Allow crab meat and 4-hexylresorcinol to equilibrate at 4°C for 18 h. To prepare spiked samples, take ca 1 g composited crab meat and add appropriate volume of 4-hexylresorcinol stock solution (1 mg/mL) to get a concentration of 20–100 μg 4-hexylresorcinol/g crab meat.

Preparation of Sample

(a) *Extraction with acetonitrile.*—Decant excess water from sample. Remove remaining moisture by blotting crab meat with absorbent towels. Shake vigorously for 10 min in a 20 mL liquid scintillation vial containing 3 mL acetonitrile/g crab meat. Decant acetonitrile extract into a volumetric flask with a capacity that is 10 times the volume of the acetonitrile extract. The crab meat becomes brittle after the first extraction and is easily broken into smaller pieces with a spatula for subsequent extractions. Repeat acetonitrile extraction 2 times with fresh volumes of acetonitrile and combine extracts. Dilute with a volume of deionized water that is 3 times the volume of the combined acetonitrile extracts. This volume is critical, because the efficiency of SPE is reduced if this ratio is less than 3:1.

(b) *Solid-phase extraction.*—Wet extraction column with 3 mL methanol and then wash with 10 mL deionized water. Pass diluted acetonitrile extract through column. Wash extraction column again with 10 mL deionized water. Elute 4-hexylresorcinol with 3 mL ethanol. Adjust volume to 3 mL with ethanol.

Analysis Conditions

Mobile phase flow rate is 1 mL/min. Injection volume is 20 μL. Detector is set at 206 nm.

Calculations

Run 9 4-hexylresorcinol working standards (0.5, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 60.0, and 80.0 μg/mL) under analysis conditions. Prepare standard curve by plotting absorbance at 206 nm vs concentration of standard. Apply linear regression analysis to data.

The calibration curve was linear from 0.00 to 80 μg/mL. The correlation coefficient was 0.9999, the slope was 685 μg/mL/absorbance unit, and the intercept was 0.000184 absorbance unit.

Extracted 4-hexylresorcinol was measured under the same conditions. Levels of the compound in extracts were determined by comparison with the calibration curve.

Calculate concentration of 4-hexylresorcinol in crab meat as follows:

$$\text{4-Hexylresorcinol, } \mu\text{g/g} = \frac{CV}{W}$$

where C is 4-hexylresorcinol concentration in extract (μg/mL), V is extract volume (3 mL), and W is weight of crab meat (g).

Results and Discussion

For this method, the water content of crab meat to be analyzed must be controlled. Crab meat samples must be blotted with reinforced absorbent towels to maintain extraction efficiency.

There is no need to homogenize the sample. Extraction with acetonitrile is efficient, and homogenization would add water to the sample, reducing extraction efficiency.

The ratio between volume of acetonitrile used and sample weight is critical. It must be 3 mL acetonitrile/g sample; otherwise extraction efficiency drops.

Samples weighing 1–4 g were used in this study. Larger or smaller sample sizes can be used depending on analytical needs. SPE produces clean extracts ready for analysis. Because the analyte is in ethanol, detection, measurement, and confirmation of 4-hexylresorcinol could be performed by LC, gas chromatography (GC) or GC/mass spectrometry without further sample preparation.

This procedure can monitor 4-hexylresorcinol in crab meat at levels between 1.0 and 100 μg/g. Data in Tables 1 and 2 show the procedure's accuracy, defined as recovery from crab meat spiked with 4-hexylresorcinol and equilibrated with 4-hexylresorcinol containing saturated sodium chloride solution. Table 1

Table 1. Recovery of 4-hexylresorcinol from equilibrated crab meat[a]

Spike level, μg/g	Recovery		Relative standard deviation, %
	μg/g	%	
20	17.4	87	6.6
10	8.8	88	3.2
1	0.93	93	9.0

[a] $n = 5$. Mean recovery = 89%. Mean RSD = 6.3.

Table 2. Individual analyses of crab meat spiked with 4-hexylresorcinol

Spike level, μg/g	Recovery, %
20	89
25	103
30	99
40	86
50	104
60	91
70	91
75	105
80	97
100	93
Mean	96
RSD	7.1

shows replicate analyses of crab meat equilibrated with 4-hexylresorcinol containing saturated sodium chloride solution. Predicted 4-hexylresorcinol values were 1, 10, and 20 μg/g. Mean recovery was 89%, with a relative standard deviation (RSD) of 6.3. Table 2 presents results of 10 individual analyses of crab meat spiked with 4-hexylresorcinol at 10 levels, ranging from 20 to 100 μg/g. Mean recovery was 96%, with an RSD of 7.1.

Figures 1, 2, and 3 show chromatograms of a 1 μg/mL standard, crab meat that does not contain 4-hexylresorcinol, and crab meat containing 4-hexylresorcinol at 20 μg/g. The procedure can detect and measure the concentration of 4-hexylresorcinol in crab meat if crab meat has comparable residual levels with those in shrimp and is processed in a manner similar to the shrimp.

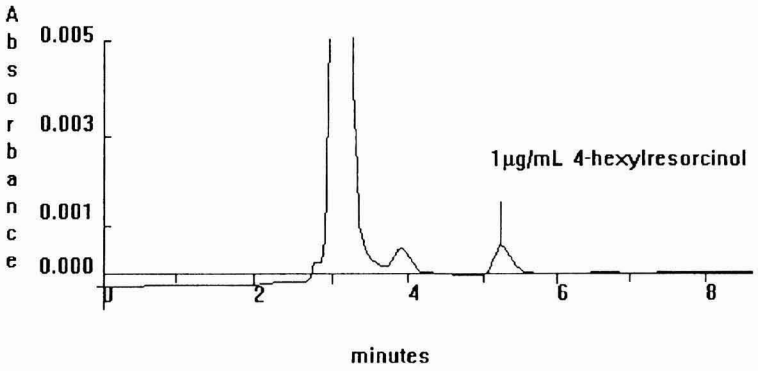

Figure 1. Chromatogram of 4-hexylresorcinol standard (1 μg/mL).

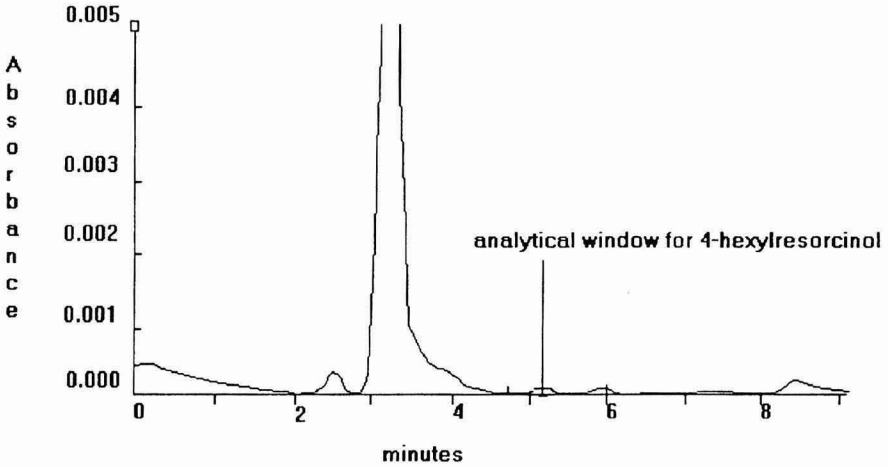

Figure 2. Chromatogram of extract from crab meat with no 4-hexylresorcinol added.

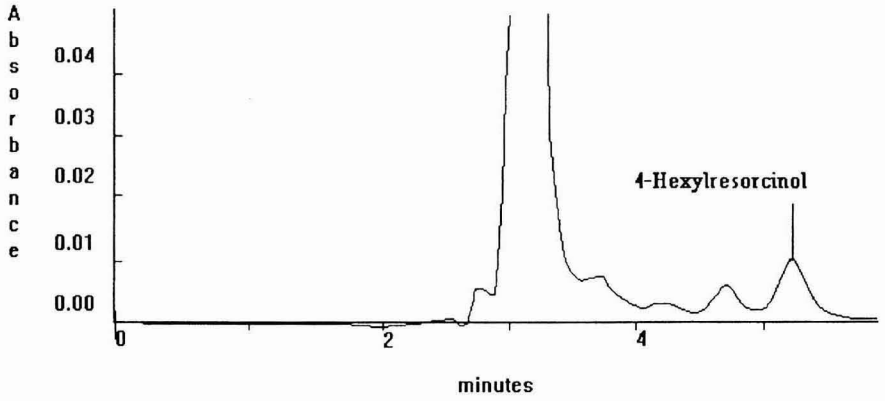

Figure 3. Chromatogram of extract from crab meat containing 20 μg 4-hexylresorcinol/g.

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Instructions to Authors

Scope of Articles and Review Process

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

General Information

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

1. Write in clear, grammatical English.

2. To Managing Editor, AOAC INTERNATIONAL, 481 N Frederick Ave, Suite 500, Gaithersburg, MD 20877, USA, submit typewritten original plus 3 photocopies (1 side only, white bond, 8½ × 11 in. [21½ × 28 cm] of complete manuscript in order as follows—*1.* Title page; *2.* Abstract; *3.* Text (introduction, method or experimental, results and/or discussion, acknowledgments, references); *4.* Figure captions; *5.* Footnotes; *6.* Tables with captions, one per page; *7.* Figures.

3. Suggest in a cover letter the names of at least 4 qualified reviewers, i.e., individuals engaged in or versed in research of the type reported.

4. **DOUBLE SPACE all typed material.** Manuscripts not double spaced will be returned for retyping. Do not right justify or use proportional spacing; avoid hyphenation.

5. Use letter quality printer for word-processed manuscripts; manuscripts prepared on dot matrix printers of less than letter quality may be refused. Once a manuscript has been accepted for publication, authors will receive instructions for submitting the final version of their accepted manuscript to AOAC on diskette. AOAC accepts MS-DOS-based files from most word processing packages or ASCII text files on MS-DOS-formatted diskettes. **(DO NOT SEND DISKETTE WITH ORIGINAL MANUSCRIPT SUBMISSION.)**

Format and Style

1. **Title page** (separate sheet, **DOUBLE SPACED**): title of article, authors' names (full first, middle initial if any, full last), authors' addresses including mail codes.

2. **Abstract** (separate sheet, **DOUBLE SPACED**): 200 words. Provide specific information, not generalized statements.

3. **Text** (consecutive sheets, **DOUBLE SPACED**): *Introduction*. Include information on why work was done, previous work done, use of compound, or process being studied.

Method or Experimental. Consult recent issue of *Journal* for proper format. Separate special reagents/apparatus from details of procedure and list in sections with appropriate headings; avoid use of brand names. (Common reagents/apparatus or those which require no special treatment need not be listed separately.) Place detailed operations in separate sections with appropriate headings (e.g., Preparation of Sample, Extraction and Cleanup). Include necessary calculations; number of significant figures must reflect accuracy of method. Use metric units for measurements of quantities wherever possible. Note hazardous and/or carcinogenic chemicals.

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

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

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

JOURNAL ARTICLE REFERENCE

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

BOOK CHAPTER REFERENCE

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

BOOK REFERENCE

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

OFFICIAL METHODS REFERENCE

(1) *Official Methods of Analysis* (1995) 16th Ed., AOAC INTERNATIONAL, Arlington, VA, secs **29.070–29.072**

4. **Figure captions** (separate sheet(s), **DOUBLE SPACED**): Designate all illustrations, including schemes, as figures and include caption for every one. Identify curves (*See* **Figures**) and include all supplementary information in caption rather than on face of figure. Spell out word Figure.

5. **Footnotes** (separate sheet(s), **DOUBLE SPACED**): Avoid use of footnotes to text. Include location/date of presentation, if appropriate; present address(es) of author(s); identification of corresponding author, if not senior author, proprietary disclaimers; institution journal series numbers.

6. **Tables** (one per page, **DOUBLE SPACED**): Refer to recent issue of *Journal* for proper layout and style, especially use of horizontal lines. Do not draw in vertical lines. Include descriptive title sufficient that table stands alone without reference to text. Provide heading for *every* vertical column. Abbreviate freely; if necessary, explain in footnotes. Indicate footnotes by lower case superscript letters in alphabetical order. Do not use one-column tables; rather, incorporate data in text.

7. **Figures**: The *Journal* does not publish straight line calibration curves; state such information in text. Do not duplicate data in tables and figures. Submit original drawings or black/white glossy photographs with original manuscript; photocopies are acceptable only for review. Prepare drawings with black India ink or with drafting tape on white tracing or graph paper printed with nonreproducible green ink. Lines, letters, numbers, and symbols must be sharp and unbroken. Use type at least 2 mm high to allow reduction to page or column size. Identify ordinate and abscissa and give value in *Journal* style (e.g., "Wavelength, nm," "Time, min"). Label curves with letters or numbers; avoid all other lettering/numbering on face of figure (*see* **Figure captions**). Identify each figure on back with number and authors' names.

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