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AOAC INTERNATIONAL

JAINEE 75(5)773-948 (1992)
ISSN 1060-3271

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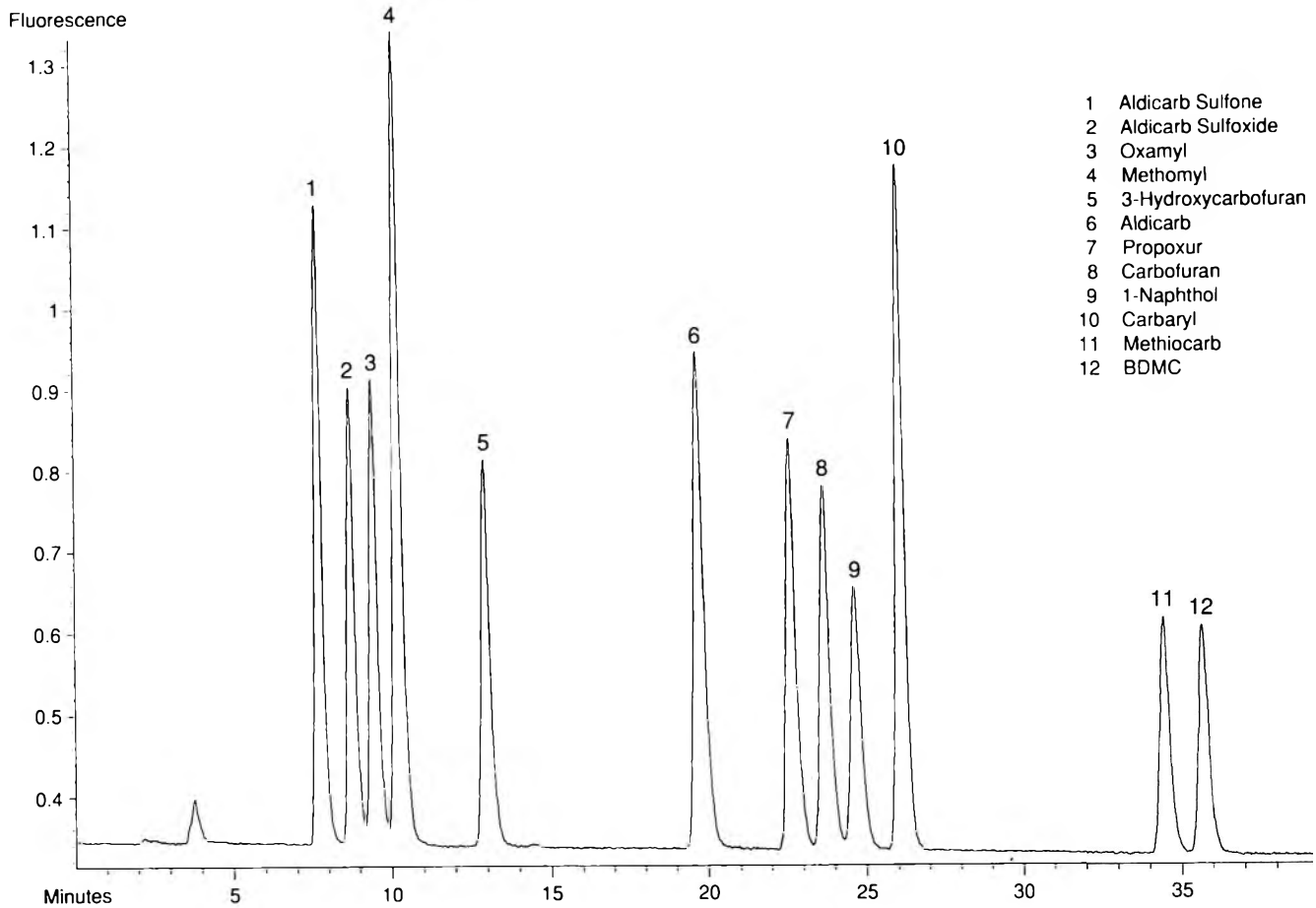

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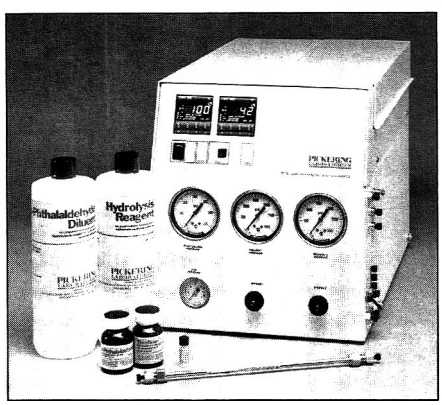

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JOURNAL OF AOAC INTERNATIONAL

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

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For Your Information

Meetings

October 8 1992: AOAC Mid-Atlantic USA Regional Section Meeting, College Park, MD. Contact: David B. MacLean, 6422 Alloway Ct, Springfield, VA 22152, telephone 703/451-1578.

October 12–17, 1992: AOAC Short Courses, St. Louis, MO. Topics: QA, Microbiological QA, Lab Waste, How to Testify as an Expert Witness, Statistics, Safety, Improving Technical Writing Skills. Contact: AOAC Meetings Dept, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

November 1992: AOAC New York/New Jersey Regional Section Meeting. Contact: Alex MacDonald, Hoffmann La Roche, Inc., 340 Kingsland St, Nutley, NJ 07110, telephone 201/235-4641.

November 4, 1992: AOAC East European Subsection Meeting, Prague, Czechoslovakia. Contact: Margreet Lauwaars, PO Box 153, 6720 AD Bennekom, The Netherlands, telephone 31 8389 18725.

November 18–19, 1992: AOAC Central USA Regional Section Meeting, Kalamzaoo, MI. Contact: Sungsoo Lee, Kellogg Co., 235 Porter St, PO Box 3423, Battle Creek, MI 49016-3423, telephone 616/961-2823.

January 21–22, 1993: AOAC Pacific Southwest Regional Section Meeting, Napa, CA. Contact: Paul Bolin, DFA of California, PO Box 86, 1855 S. Van Ness, Fresno, CA 93721, telephone 209/233-7249.

January 31–February 2, 1993: AOAC Southwest USA Regional Section Meeting, Little Rock, AR. Contact: Thomas Wilson, Colonial Sugars, Inc., PO Box 3360, Gramercy, LA 70052-3360, telephone 504/869-5521.

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

March 29–30, 1993: AOAC Europe Regional Section Meeting, Barcelona, Spain. Contact: J. Sabater, Laboratorio Dr. J. Sabater Tobella, Calle de Londres

6, 08029 Barcelona, Spain, telephone 343-32288 06.

March, 1993: AOAC MidAtlantic USA Regional Section Meeting. Contact: David B. MacLean, 6422 Alloway Ct, Springfield, VA 22152, telephone 703/451-1578.

AOAC Official Methods Board News

The AOAC Official Methods Board met May 28–30, 1992, in St. John's, Newfoundland. At that meeting, the Board reviewed and adopted seven methods as first action AOAC Official Methods (previously listed in the July 1992 issue of *The Referee*).

As directed by the Board of Directors, the Official Methods Board is also responsible for approving recommendations by General Referees and Methods Committees for repealing or declaring surplus AOAC Official Methods. Notification of proposed actions were listed in previous issues of *The Referee*, actions were taken as listed below at the May 28–30 Official Methods Board meeting, and these actions became official at that time. These actions will be published in the fourth supplement (1993) to the 15th edition (1990) of *Official Methods of Analysis*. Results of the 1992 membership vote on adoption of recommended first action methods as final action will also be published in the fourth supplement.

Methods Voted for Surplus Status

Pesticide Formulations and Disinfectants

977.05 Methyl Parathion in Pesticide Formulations, Liquid Chromatographic Method (First Action)

978.07 Parathion in Pesticide Formulations, Liquid Chromatographic Method (First Action)

985.07 Fenitrothion Technical and Pesticide Formulations, Gas Chromatographic Method (Final Action)

Drugs and Related Topics

962.23 Santonin in Drug Mixtures, Ultraviolet Absorption Method (First Action)

968.48 Ethoxyquin Residues in Animal Tissues, Photofluorometric Method (First Action)

Foods I

960.37C(b) Plant Material (Foreign) in Vanilla Extract, Paper Chromatographic Method, Preparation of Authentic Vanilla Extract, FEMA method (Final Action)

Foods II

967.17 Anthocyanins in Fruit Juices, Paper Chromatographic Method (Final Action)

968.21 Malvidin Glucosides in Grape Juice, Paper Chromatographic Method (Final Action)

969.30 Organic Acids (Foreign) in Fruit Juices, Paper Chromatographic Method (Final Action)

Methods Repealed

Methods that have proved unsatisfactory or that have been superseded by a superior method may be recommended for repeal by the appropriate General Referee. Repealed methods lose their status within the organization and are deleted from *Official Methods of Analysis*. The following General Referee recommendations were approved:

Pesticide Formulations and Disinfectants

964.04 DDVP in Pesticide Formulations, Infrared Spectrophotometric Method

966.07 DDVP in Pesticide Formulations, Infrared Spectrophotometric Method

Microbiology and Extraneous Materials

985.42 *Salmonella* in Foods, Hydrophobic Grid Membrane Filter Screening Method

Books in Brief

Element-Specific Chromatographic Detection by Atomic Emission Spectroscopy. Edited by Peter C. Uden. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1992. 345 pp. Price: $74.95. ISBN 0-8412-2174-X.

Here is the first book to specifically cover interfaced chromatographic separations and element-specific detection by AES. The volume offers fundamental research studies and reviews by the most active groups in the field, including those involved in instrument development. A wide variety of techniques are discussed, including LC/GC, SFC, atomic emission spectroscopy, plasma spectroscopy, microwave induced plasmas, inductively coupled plasmas, direct current plasmas, and inductively coupled plasma/mass spectroscopy. This book serves as a valuable source of information for analytical and environmental chemists, toxicologists, medicinal and pharmaceutical chemists, and instrument developers.

Accountability in Research: Policies and Quality Assurance. A Gordon and Breach journal. Edited by Adil E. Shamoo, Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, MD. Published by Gordon and Breach Science Publishers, PO Box 786, Cooper Station, New York, NY 10276. 4 issues per volume. Base list rate per volume: $76.00. Corporate library rate per volume: $215.00. ISBN 0898-9621.

Increasingly complex research, increased time and distance between the steps of gathering, manipulating, and analyzing data, and financial pressures have led to an impression of lower than expected quality of research data... whether a result of unintentional error or intentional fraudulent activity. Focusing on the entire subject, this journal is devoted to the study, critical analysis, and examination of systems for conducting research. It is an international, interdisciplinary forum for the development of

new policies, procedures and standard for assuring the integrity of research data.

Concepts & Calculations in Analytical Chemistry: A Spreadsheet Approach. By Henry Freiser. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1992. 315 pp. Price: $19.95. ISBN 0-8493-4717-3.

A major objective of this book is to help you become familiar with and confident about the important ideas used by analytical chemists. To accomplish this, all tedious and repetitive arithmetic operations are conquered with the help of a computer spreadsheet program called QuattroPro (QPro). References in the text are given to the QPro menu and commands, but equivalent menus, such as those for Lotus 123 or their older Quattro versions, can be called up. This book is not a spreadsheet manual, however. It is an analytical chemistry text that derives benefit from the liberal use of the spreadsheet approach. What you learn here will have a bearing in problem solving not only in chemistry but also in most other sciences.

Biosensors. By F. Scheller and F. Schubert. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1992. 360 pp. Price: $161.50. ISBN 0-444-98783-5.

Biosensor research, development, and applications are surveyed in this book from the perspective of current and future directions. The basic principles of the coupling of molecular recognition with signal processing in biosensors are presented to any interdisciplinary readership in such a way as to achieve an understanding of the biochemical and technological peculiarities of that functional unit of microelectronics and biotechnology that is called a biosensor. Two main classes are distinguished according to the type of interaction leading to signal generation: metabolism sensors and affinity sensors. The technological aspect is stressed by discussing the sensors according to their inherent degree of integration.

Xenobiotics and Food-Producing Animals: Metabolism and Residues. Edited by D.H. Hutson, D.R. Hawkins, G.D. Paulson, and C.B. Struble. Published by the American Chemical Society, 1155 16 St, NW, Washington, DC 20036, 1992. 240 pp. Price: $58.95. ISBN 0-8412-2472-2.

This volume discusses the metabolism and fate of xenobiotic compounds, such as veterinary drugs, agrochemicals, and other products to which food-producing animals are exposed. It describes state-of-the-art techniques for experimental studies of xenobiotic compounds in ruminants, poultry, and aquatic species, including study design to meet specific regulatory requirements.

Biotechnology and Food Ingredients. Edited by Israel Goldberg and Richard A. Williams. Published by Van Nostrand Reinhold, PO Box 668, Florence, KY 41022-0668, 1991. 577 pp. Price: $99.95. ISBN 0-442-00272-6.

Here is the first reference book dealing specifically with the development of food ingredients through biotechnological processes. Industry experts explain how to produce proteins, vitamins, enzymes, flavors, colors, and many other ingredients using leading edge techniques. Several of the methods discussed reflect advances in genetic engineering or entirely new biotechnological processes. Strategies for customizing ingredients and raw materials that improve the food supply and meet consumer needs make this a valuable source for scientists and researchers in the food processing and ingredient industries, as well as academic and government researchers.

New Products

Computer Based Training Packages

A series of computer based training packages has been developed to provide training, continuing education, and updating in 7 of the most widely used techniques of analytical science. These training packages are in addition to the very successful well-established series of 30 open learning books in the ACOL series published by John Wiley & Sons. These computer based packages are designed to be useful to those with some experience as well as those new to a technique. Each package consists of a computer based training program on disk and an open learning book. The computer program provides experience of the many decisions that have to be made during an analysis and is aimed to be used after studying the text or by those already conversant with the basic theory. The program offers the user the choice of instrument configurations and other variables such as calibration standards, pH, flow rate, stationary phase, wavelength, and attenuation. The outcome of each choice, together with relevant reinforcement or guidance, if necessary, is seen immediately on the computer screen, often simulating instrument output. Thames Polytechnic.
Circle No. 348 on reader service card.

Eppendorf Repeater Pipet

The Eppendorf Repeater pipet dispenses up to 48 samples at 1 s intervals without refilling. The Repeater pipet features a unique Eppendorf Combitip reservoir that provides repetitive pipetting. You can chose any of 22 different volumes from 10 μL to 5 mL, and the Repeater volume dial allows selection of 5 different volumes for each Combitip size. The Repeater pipet is ideal for use with aggressive or viscous liquids, because reagents contact only the Combitip tip and not the instrument itself. Bio-Rad Laboratories.
Circle No. 349 on reader service card.

Monitor Nitrate On-Site with New Electrode

A combination nitrate electrode measures nitrate between 0.10 and 14 000 mg/L to help identify water contamination. The electrode is fast, accurate, and convenient, providing 95% of response in less than 30 s at nitrate levels above 2 mg/L. The maximum contaminant level for nitrate has been established at 10 mg/L by the U.S. Environmental Protection Agency. The electrode is compatible with any Hach One pH/mV or pH/ISE meter, and any meter using a BNC connector. Hach Co.
Circle No. 350 on reader service card.

Compression Module Applies Pressure to Chromatographic Cartridges

The Kiloprep 100 benchtop compression module (KPCM 100) applies dynamic, self-compensating pressure to chromatographic cartridges. By applying radial compression to the outer surface of either a 3 in. × 15 or 30 cm prepackaged cartridge, the KPCM 100 is capable of greatly reducing band spreading and provides high efficiency and increased reliability. The use of prepacked cartridges eliminates the need for time consuming packing and testing of comparable steel columns and allows cartridge replacement to be achieved in minutes. Biotage, Inc.
Circle No. 351 on reader service card.

New Family of HPLC Pumps Support Gradient and Isocratic Applications

The SpectraSystem P1000, P1500, P2000, and P4000 provide easy-to-use menus, dual in-line pistons, patented ceramic check valves, and stainless or inert (PEEK) solvent paths. The P2000 and P4000 pumps provide accurate and reproducible gradients and low rates. The P4000 includes the use of 4 solvents, 11 user files, a "Develop File" for method development, and 21 gradient curves. The P2000 includes the use of 2 solvents and 4 named files. Both these pumps are multi-tasking, allowing operating conditions to be changed in real time while the pump is running. The P1500 and P1000 provide for reliable and reproducible isocratic chromatographic separations. The P1500 allows use of 2 solvents for step gradients or system cleanup. The P1000 is a single-solvent pump. Spectra-Physics Analytical.
Circle No. 352 on reader service card.

Dual Observation Tube Available for Zeiss Standard Microscope Series

An affordable, high-quality dual observation tube is now available for microscopes in the Zeiss Standard 16, 20, or 25 series. This highly functional unit is ideal for a wide range of teaching, research, and clinical applications. It can be installed in seconds and is easy and convenient to use. Carl Zeiss, Inc.
Circle No. 353 on reader service card.

Acquire, Analyze, and Present Chromatographic Data in Full Color or at Full Dot Resolution

Peaks Version 1.1 for the Apple Macintosh allows you to acquire, analyze, and present your chromatographic data in full color, or at the full dot resolution of your Laser Writer printer. PEAKS software allow export of pictures as well as computed values, such as height and area to spreadsheets, word processors, graphics programs, and databases. PEAKs can be used as a software only analysis package, operating on any chromatogram saved as an ASCII file.

Or, using the MacLab Data Interface Module, as many as 4 detector channels can be recorded directly to your Macintosh computer. The MacLab Data Interface Module is as easy to use as a chart recorder, and it runs in the background so you can use your Mac to analyze data or work in other applications while data is being recorded. MacLab Division, ADInstruments, Inc.
Circle No. 354 on reader service card.

Data Management Made Easy

The EM Science Chemical Information System (EMCIS) is an extremely flexible database that provides laboratories with cross-referenced regulatory, storage, disposal, DOT shipping information, MSDS, worker safety and emergency response information, as well as physical and chemical properties data on over 4000 products. Additionally, it features suggestions for safe cleanup procedures, EPA disposal requirements, and methods for properly handling a wide variety of substances. EM Science offers quarterly updates on all safety and regulatory information to help assure that laboratories always have the most accurate, reliable information available. The EMCIS database is expandable. Because it employs Microsoft Windows 3.0 software, EMCIS provides a framework that makes it easy for laboratories to input their own data into the system, thus creating their own customized information sources. EM Science.
Circle No. 355 on reader service card.

Aromatic Analzyer Replaces FIA for Determination of Aromatic Content of Petroleum Fuels

The Aromatic Analzyer, a dedicated analysis system for the determination of the aromatic content of petroleum fuels, replaces current fluorescent indicator adsorption methods. It provides excellent quantitation of the total aromatic content of diesel fuels, jet fuels, gasoline, and other types of petroleum products. The analysis methodology is based on ASTM Method No. D5186 for the analysis of the aromatic content in diesel fuel, which has proven round-robin, intralaboratory reproducibilities over a wide range of aromatic content values. Suprex Corp.
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Ultra Pure Nitrogen Gas from Compressed Air

A compact nitrogen gas generation system produces up to 550 cc/min of compressed nitrogen on site at purities of up to 99.9995%. This system uses several new technologies that separate the gas components from compressed air to produce high purity nitrogen gas at safe, regulated pressures without operator attention. Typical applications include: chemical and solvent blanketing, glove box purge, chemical and solvent evaporation, instrument purge and supply, evaporative light scattering detector (HPLC), and sparging. Balston Inc.
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Pour Plates for Safe Quenching of Fusions or Fluid Melts

Pour plates provide a safe heat-sink for quenching fusions or pouring fluid melts to solidify into a button for rapid, easy handling and solution. Alkali metal carbonates, bisulfates, or lithium fluxes are readily employed without contamination. The pour plates provide safe handling of carbonate or bisulfate fluxings in platinum-water cleaning. Pour plates are available in stainless steel and copper, machined from solid metal plate. Size is 6 in. diameter and 1/4 in. thickness. B-J Scientific Products, Inc.
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Portable, Compact 12-Volt Battery Pack System

A portable, compact yet economical 12-volt battery pack provides up to 18 h of power to rock, shake, or stir cell cultures or experiments in the field or in the laboratory. Ideal for use with 12-volt rocker platforms, orbital shakers, reciprocating shakers, and magnetic stirrers manufactured by Bellco, these 12-volt systems offer inexpensive protection against power failures and loss of agitation that could ruin cell cultures and experiments. Bellco Glass, Inc.
Circle No. 359 on reader service card.

Extractor/Concentrator Saves Time, Cuts Costs

An environmental extractor/concentrator apparatus saves time and cuts costs by allowing extraction and concentration in a single unit. The Pyrex One-Step Soxhlet extractor/concentrator body assembly requires only one setup and one cleanup, reducing turnover time and cost per analysis. There is no sample transfer between extraction and concentration, decreasing chances of contamination. The unit is made from Pyrex borosilicate glass that resists thermal expansion and chemical corrosion. It is suitable for semi-volatile and non-volatile samples and solid and semi-solid samples as dictated by SW-846, Method 3540. Standard joint sizes permit it to be used with existing condensers and flasks. Corning Inc.
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Safety Barrier System Cuts Cost and Space

The Elcon Series 1600 combines the proven performance of a zener barrier with the convenience of plug-in technology, eliminating the need for separate cable terminations. With up to 96 barrier channels in a 19 in. space, the Series 1600 is a full featured line of safety barriers suitable for protecting a wide variety of hazardous area instrumentation. Housing up to 6 barrier circuits per module, the plug-in design permits removal of barriers without disturbing the ground connection or any potentially dangerous wires. This allows the user to simply and safely replace the individual barrier protecting fuses.
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Laboratory Furniture Modules

LabExpress is designed to provide everything needed to build your own in-house laboratory from start to finish. It includes simplified planning, layout, and ordering procedures; from stock availability of selected Labx furniture modules; and easy-to-follow assembly instructions. Each Labx module (31 ½ in. × 31 ½ in.) comes complete with its own work service, storage system, and self-contained utilities. Modules can be joined together to form work areas along walls, as peninsulas, or as free standing islands. Captairlabx.
Circle No. 362 on reader service card.

Screw Thread Polypropylene Limited Volume Vial

A polypropylene 0.75 mL, 12 × 32mm limited volume vial with a 10 mm/425 screw threat is ideal for use with drug and protein samples that adhere to glass, samples which are sensitive to pH changes or ion contamination, and infectious and toxic samples. The ID opening is 50% larger than typical glass vials, permitting easy filling and decanting, and the unique cone-shaped bottom allows maximum sample evacuation. J.G. Finneran Associates, Inc.
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Point of Use Recirculation for Deionization Systems

Designed for service deionization units from 0.25 to 1.20 cubic foot, the Ionpure Plus 150 wall-mounted recirculation system will deliver up to 8 L/min of 18 + megohm water. The feedwater enters the system and after passing through a prefilter cartridge, passes through a series of portable vessels that typically contain activated carbon. One or 2 tanks containing mixed bed resins then polish the water. Ionpure.
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Letter to the Editor

Composition of Pineapple Juice

In the March/April 1992 issue of the *Journal of AOAC International* (Vol. 75, No. 2, pp. 280–282), there appeared an article by Dana A. Krueger and co-authors. It contains omissions, factual errors, and strange and unstatistical data. I do not know if I am more disturbed at Krueger for his omissions, inaccuracies, and unstatistical data or at the referees who allowed this to be published.

I will begin by taking exception to Krueger's statement that the literature on pineapple is meager. The U.S. Food and Drug Administration began analyzing sugars in pineapple in 1970 (1), and Michigan State University (2) began in 1981. The Health Protection Branch Canada (3) and the U.S. Department of Agriculture (4) both reported organic acids in pineapple in 1973. I reported on major components in pineapple at a workshop on the detection of juice adulteration (5) in 1990 in Washington, DC, which was attended by Krueger. Along with the data cited by Krueger in those publications he did find (6–12), it would seem that Krueger's description of the literature on pineapple as meager is somewhat inaccurate.

I have summarized Krueger's data in Table 1. In addition, I have calculated standard deviations (δ) and variances (CV).

The major type of pineapple product encountered in the commercial marketplace is canned pineapple juice, which is generally made from concentrate (21CFR146.185) (13). CFR defines minimum Brix levels for fresh squeezed as well as juice from concentrate, acidity levels (as critic), and Brix/acid ratio minimums. The Brix/acid minimum for pineapple juice is 12.0 per 21CFR146.185 b (iii). Three of Krueger's samples fall below this level and, thus, do not meet minimum standards of identity (20% of his samples, thus, are invalid).

E.R. Elkins et al.(8) accurately note that 74% of the world's pineapple supply originates in the Philippines and Thailand. Only 2 of Krueger's 15 samples (13%) were from these countries. Eleven were from Hawaii and Costa Rica, which is not exactly representative of the world marketplace.

Krueger states that pineapple prices have gone up due to a decline in production. Industry sources (14, 15) explain that production has risen each year but demand has exceeded supply, which under the laws of supply and demand usually leads to price increases. Thus, product shortage, not production decline, is the cause of the price rise. Pineapple export figures are shown in Table 2 (concentrate is the major form of export in juices).

Next, a discussion of Krueger's unusual data is in order. Three of his sam-

Table 1. Krueger data

	BRIX	TA[a]	B/A[b]	C[c]	M[d]	F[e]	G[f]	F/G[g]	S[h]	S/TS[i]	C/M[j]	IC[k]	C/IC[l]	$\delta^{13}C$[m]	K[n]
H1	15.5	.14	13.6	1151	291	2.12	1.81	1.17	9.73	71	3.96	219	52	−12.1	1410
H2	11.7	0.89	13.1	876	210	1.72	1.44	1.19	6.32	67	4.17	158	55	−11.2	890
H3	13.5	1.21	11.2	1103	199	2.23	1.21	1.84	8.14	70	5.54	255	42	−11.3	NR
H4	11.2	0.96	11.7	907	253	2.41	1.33	1.81	5.58	60	3.58	231	39	−11.2	NR
H5	13.0	0.53	24.5	535	102	3.30	2.53	1.30	5.56	48	5.25	165	32	−11.4	1350
H6	14.4	0.66	21.8	597	73	3.50	2.52	1.38	6.30	51	8.18	156	38	−11.5	830
C1	14.9	1.00	14.9	825	391	2.57	2.29	1.12	8.63	64	2.10	165	50	−12.8	1110
C2	14.1	1.21	11.7	796	172	5.05	4.52	1.12	3.10	24	4.62	196	41	−12.7	NR
C3	14.6	1.01	14.6	929	234	3.26	2.92	1.13	6.51	51	3.97	170	55	−12.4	NR
C4	16.2	0.46	35.2	505	86	3.73	3.49	1.07	7.56	51	5.87	112	45	−12.0	1320
C5	13.6	1.06	12.8	955	222	2.46	1.96	1.26	6.11	58	4.30	181	53	−13.1	1390
D	12.4	0.49	25.3	456	151	4.37	4.04	1.08	2.47	23	3.02	127	36	−13.0	1350
P	13.2	0.68	19.4	439	182	4.14	4.18	0.99	2.81	25	2.41	94	47	−12.5	1150
T	14.1	0.46	30.7	557	143	3.36	3.71	0.91	6.53	48	3.90	108	52	−12.4	1340
U	14.1	0.72	19.6	540	297	2.23	2.62	0.85	8.39	63	1.82	80	58	−13.5	1020
$\bar{X}$	13.8	0.83	18.0	745	200	3.10	2.70	1.21	6.25	52	4.18	162	47	−12.2	1196
δ	1.3	0.27	8.3	231	83	0.92	1.04	0.27	2.08	16	1.57	51	9	0.7	199
CV	9	32	46	30	42	30	39	22	33	31	38	31	19	6	17

[a] Acidity, g/100 mL.
[b] BRIX/acidity.
[c] Citric, g/100 mL.
[d] Malic, g/100 mL.
[e] Frutose, g/100 mL.
[f] Glucose, g/100 mL.
[g] Frutose/glucose.
[h] Sucrose, g/100 mL.
[i] Sucrose/TS.
[j] Citric/malic.
[k] Isocitric, mg/L.
[l] Citric/isocitric.
[m] PDB, ‰VS.
[n] Potassium, mg/L.

Table 2. Worldwide pineapple exports, concentrate, 1000 tons × 10^3

	1988	1989	1990	1991 (Estimated)
Philippines	29.0	32.5	40.1	43.0
Thailand	40.2	58.5	71.0	75.0
Kenya	10.4	8.2	10.0	N/A
Brazil	11.3	11.8	6.7	N/A
South Africa	5.6	7.4	9.8	N/A
Totals	96	118	138	142
% from Philippines and Thailand	82%	77%	80%	80%

ples (H3, H4, C2, see Table 1) do not meet the FDA/USDA standards of identity (B/A minimum 12.0), hence, they could not legally be sold as pineapple juice. Three of his samples (C2, D, P) have obviously inverted, although he chooses not to notice this. Inversion in processed pineapple is relatively rare because the heat used to concentrate pineapple juice usually inactivates any enzymes that might cause inversion. However, Krueger's samples were fresh pressed; thus, inversion becomes possible and obviously occurred in these 3 samples. It is regrettable that he does not seem to recognize that inversion has occurred. His only comment is that low acid pineapples, described as very ripe (old?), may have undergone hydrolysis. Unfortunately, one of the 3 samples that hydrolyzed, C2, had the highest observed acid level at 1.21%. Although his sample D had a low acid level at 0.49%, samples T and C4 were even lower at 0.46% but did not show any inversion. His explanation seems inadequate in my opinion. Quite frankly, it is just as possible that all 3 samples had spoiled microbiologically during, or prior to, assay; yeast is known to be high in invertase. Because these samples are so different in chemical values, it is my opinion that they should not have been included in this study. In fact, elevated fructose and glucose levels, along with low sucrose levels, are most frequently observed when invert sugar has been deliberately added to the sample. Thus, adulterators can use such faulty samples as justification for their deception.

Now, there is the question of obvious mistakes. We assume, of course, they are just typographical errors, but the units for isocitric are given as g/L. These should be mg/L (which is the equivalent of ppm). The arithmetic mean of the potassium values given in Krueger's Table 3 is 1196, not 1100. Why did they only run 11 of the 15 samples?

Next, there is the question of unusual values. Samples H1 and H3 show citric levels of >1100 mg/dcl, and sample C1 shows a malic value of 391 mg/dcl. No one else has ever observed such levels.

Table 3. Brause 1990 guidelines

% Fructose	1.4–3.3	Isocitric	>60 ppm
% Glucose	1.2–2.8	Citric/malic	3.0–5.3
% Sucrose	6.0–8.0	Citric/isocitric	30–108
% Fructose/glucose	1.00 ± 0.1	$\delta^{13C\ Juice}$	−12.0 to −15.0
%Sucrose/total sugars	53–75%	$\delta^{3C\ Citrate}$	−12.0 to −15.0
mg/100 mL Malic	100–225	Potassium	>1000 ppm
mg/100 mL Citric	450–650	Sodium	<50 ppm

I am unaware of anyone who has observed citric or malic at levels of >900 and >300 mg/dcl, respectively. These unusual values, along with some uniquely low levels of citric (D, P) and malic (H6, C4), cause his variances of these parameters to be 30 and 42%, respectively. His sugar levels showed 30, 39, and 33% variance for fructose, glucose, and sucrose, respectively. Such large variances, I feel, are a cause for concern. My understanding of statistics is that variances greater than 20% are unacceptable.

Two last points remain. Krueger reports that the liquid chromatographic level of citric and malic, when added together, is substantially higher than the titrable acidity level. At first glance, this seems like an error because, in most fruit, the T.A. is higher than the total determined by liquid chromatography (4); normally, other acids are present besides those that are mainly characterized. But, again, Krueger either did not notice this or chose not to comment. This could be explained by the fact that pineapple has malic and citric present not only as acids but also as salts (buffers). In the AOAC assay method (986.13) (16), the mobile phase is pH 2.5; any acid salts present will be converted to the acids. Thus, higher levels of citric and malic as determined by liquid chromatography (vs T.A.) in pineapple are observed. Finally, Krueger takes Elkins' work to task for possibly being contaminated by apple juice. Apple juice is known to have fructose/glucose levels of >2.00 (17, 18) and high malic acid levels (9). Elkins' samples showed neither elevated fructose/glucose nor increased malic levels; hence, Krueger's suggestion that Elkins' samples had apple present seems unwarranted. Krueger says that the finding of sorbitol could cast some doubt on the validity of their results. In fact, their results, translated to single strength juice (they were reported as concentrates), look just like my 1990 criteria (means:

fructose 2.02, glucose 2.14, sucrose 6.29, malic 243, citric 692, IC 58). Such criteria came mainly from the literature (1–4, 12, 19).

In summary, I think publication of this data was a grievous error given all the seeming omissions, errors, and inaccuracies contained therein. The problem is once we give such material an official stamp of approval, it is nearly impossible to repair the damage.

I have one suggestion for the future to try to prevent reoccurrences. The reviewers may not have been that knowledgeable about pineapple. The Technical Committee for Juice and Juice Products contains members who are experts on all juices. Perhaps, in the future, papers on juice composition could be referred to this Technical Committee to suggest reviewers that do possess such specific content knowledge.

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Reaction to Letter to Editor on Composition of Pineapple Juice

Brause makes a number of criticisms of our paper entitled "Composition of Pineapple Juice" in his letter to the *Journal of AOAC International*. He describes the paper as containing numerous "factual errors," "omissions," and "strange and unstatistical data" (sic). As a matter of style, his language is offensive and borders on libelous. His criticisms are largely trivial or mistaken; they will be discussed in turn below.

Brause takes issue with the comprehensiveness of the literature cited in the paper. He cites 5 references that he feels should have been cited in the paper; 2 of these citations are in fact erroneously cited in his letter (Editor's note: corrected in editing process). The papers of Kline et al. ([1970] *J. Assoc. Off. Anal. Chem.* **53**, 1198–1202) and Richmond et al. ([1981] *J. Agric. Food Chem.* **29**, 4–7) each present sugar analyses of one sample of pineapple juice. The papers of Ryan and DuPont ([1973] *J. Agric. Food Chem.* **21** 45–49) and Chan et al. ([1973] *J. Agric. Food Chem.* **21**, 208–211) present citric and malic acid analyses for one and 2 samples of pineapple juice, respectively. A more thorough literature survey would have cited these papers, but the data that they contain does nothing to alter our assertion in the introduction of our paper that the available data in the literature on the composition of pineapple juice is meager. The results in these papers are consistent with the results in our study, with the exception of the 2 citric acid values in Chan et al., which are lower than we observed. The compositional ranges that Brause puts forward as his own have not been published and in our opinion do not merit citation.

Brause's remarks regarding the causes of the high price of pineapple juice are quibbles, and are not relevant to our study.

Brause indicates that some of our samples do not meet U.S. Food and Drug Administration Brix/acid ratio standards. This is true and irrelevant to the subject of the study.

Brause indicates dissatisfaction with the sugar results for 3 samples; he says that they "have obviously inverted;" he speculates that the samples may have been microbiologically spoiled and refers to the samples as "faulty." The data

for these samples were generated promptly following pressing; the samples were not fermented. That Brause dislikes the range of sugar values exhibited by pineapples is no concern of ours. His observation that the addition of invert sugar will also lower the sucrose content has no relevance to our study.

We were also surprised by the low sucrose values in some of the samples. In a preliminary draft of the manuscript, we observed a correlation between sucrose content and acidity; when the last few data had been collected, this correlation had largely disappeared. The 2 sentences of our paper suggesting such a correlation are in error; the 2 sentences are an inadvertent relic of an earlier draft.

Brause correctly points out a typesetting error in the heading of Table 2, where the units for isocitric acid should be mg/L; the units are referred to correctly in the text. More significantly, Brause correctly points out an error in the potassium data; the mean value referred to in Table 3 and in the text should be 1200 mg/L, not 1100 mg/L. The query of Brause as to why we did not analyze for potassium in 4 samples is impertinent.

The remarks of Brause regarding the "unacceptable" character of the variances of the data for sugars and acids are more a statement of his understanding of statistics than a criticism of our data. If nature produces a wider variance than Brause likes, that is his problem.

Brause objects that "he is unaware of anyone else" observing levels of malic and citric acid as high as reported in one of our samples. Because there are only a few valid data in the published literature for these 2 parameters, it is not surprising that our range is wider than other published data. Brause also refers to 2 "uniquely low" levels of citric acid that we find; evidently he has not noted that Chan et al. found levels of citric acid lower than any that we found. In fact, one of the 2 samples to which he objects had citric acid levels within the range that he himself asserts as normal.

That the sum of malic and citric acids exceeds the titratable acidity is neither surprising nor particularly noteworthy. The sum of enzymatic or LC nonvolatile acid results exceeds the titratable acidity for many fruit juices; this is due to the fact that the nonvolatile acids are partially neutralized as potassium salts in the juice. The data of Ryan and DuPont, cited by Brause, demonstrates this in 15 of 18 fruit juice samples analyzed, including pineapple. Brause is apparently unaware of this aspect of fundamental fruit juice chemistry.

Contrary to the assertions of Brause, we do not "take to task" the work of Elkins et al. We discussed their work at face value, only expressing reservations relating to the statement made by Heuser, not by us, that the samples they analyzed may contain apple juice. The normalized results that he attributes to Elkins et al. based on their pineapple concentrate data are simply erroneous. Although his normalization factor is inconsistent from parameter to parameter, he appears to be trying to normalize the data from 72 Brix to 12 Brix. If properly normalized from 70 Brix (the value stated in the paper) to 14 Brix (the average Brix value for pineapple juice according to our work as well as that of Sale and of Gebhart et al.), the organic acid data of Elkins et al. is very similar to our own.

The "Brause 1990 guidelines" that Brause puts forward require some comment. Brause asserts that these criteria "came mainly from the literature." This statement is plainly and unambiguously false; he cannot cite any piece of peer reviewed literature that promulgates any of these ranges. Most of the published literature on the composition of pineapple juice plainly contradicts them. His sucrose range is contradicted by Richmond et al., by Elkins et al., and by our own work. His guideline for fructose/glucose ratio is contradicted by Kline et al., by Richmond et al., by Elkins et al., and by ourselves. His ranges for citric and malic acids are contradicted by Chan et al., by Elkins et al., and by ourselves. His range for citric/malic ratio is contradicted by Chan et al. and by ourselves. There is no published data on isocitric acid levels as low as 60 mg/L; the lowest published value is our own at 80 mg/L. The work of Krueger et al. and our present work plainly refutes his carbon isotope ratio range. The paper that he cites by Doner in support of his range for citrate carbon isotope ratio has nothing whatever to do with pineapple juice; the paper is about analysis of lemon juice. There is nothing in the literature on this parameter in pineapple juice. His range for potassium is contradicted by our work, which is the only paper presenting ranges of values for potassium in pineapple juice, rather than merely mean values. His sodium range is clearly inapplicable to reconstituted pineapple juice. In short, his guidelines bear no correlation to the composition of pure pineapple juice; his suggested ranges, unsupported by data, are in our experience not typical of ranges for pure commercial pineapple juice.

In summary, we agree with Brause that the units for isocitric acid in Table 2 should be corrected, that the mean value for potassium should be corrected, and that the reference to a correlation between sucrose content and acidity should be deleted; these errors are self evident from a careful reading of the paper. The other criticisms of Brause are either mistaken or irrelevant, and do not warrant serious consideration.

–Dana A. Krueger
–Rae-Gabrielle Krueger
–Jeanne Maciel
Krueger Food Laboratories, Inc.

This is the fifth annual report summarizing the results of pesticide residue monitoring by the Food and Drug Administration (FDA). The 4 previous reports, published in the Journal of the Association of Official Analytical Chemists, *presented findings from Fiscal Years (FY) 1987 through 1990. This report includes findings for FY91 (October 1, 1990 through September 30, 1991). FDA's monitoring over these years continues to indicate that the levels of pesticide residues in the U.S. food supply are generally below established safety limits.*

RESIDUE MONITORING 1991

The responsibility for the regulation of pesticides is shared by 3 federal government agencies. The Environmental Protection Agency (EPA) registers or approves the use of pesticides and sets tolerances (the maximum amount of a residue that is permitted in or on a food) if use of that particular pesticide may result in residues in or on food. Except for meat, poultry, and certain egg products, for which the Food Safety and Inspection Service of the U.S. Department of Agriculture (USDA) is responsible, FDA enforces tolerances in imported food and in domestic food shipped in interstate commerce, acquires incidence/level data on designated commodity/pesticide combinations, and carries out the Total Diet Study. USDA's Agricultural Marketing Service has initiated a residue testing program of raw agricultural products intended to complement existing federal programs.

FDA MONITORING PROGRAM

Regulatory Monitoring

Under this approach, FDA samples individual lots of domestically produced and imported foods and analyzes them for pesticide residues. Domestic samples are collected as close as possible to the point of production in the food chain; import foods are collected at the point of entry into U.S. commerce. Fresh produce is analyzed as the unwashed whole, raw commodity, i.e., with the peel or skin intact. If illegal residues (above EPA tolerance or no tolerance for that particular food/chemical combination) are found in domestic samples, FDA can invoke various sanctions, such as a seizure or injunction. For imports, shipments may be stopped at the port of entry when illegal residues are found. "Automatic detention" may be declared for imports if one violative shipment is found and there is reason to believe that the same conditions will occur in future lots during the same shipping season for a particular grower, shipper, geographic area, or country.

The food samples that are collected are classified as either "surveillance" or "compliance." Most samples collected by FDA are the surveillance type; i.e., there is no prior knowledge or evidence that a specific food shipment contains illegal pesticide residues. Compliance samples are taken as follow-up to the finding of an illegal residue or when there is evidence of a residue problem.

▼ Analytical Methods

To analyze the large numbers of samples whose pesticide treatment history is usually unknown, analytical methods capable of simultaneously determining a number of pesticide residues are usually used. The multiresidue methods (MRMs) most commonly employed by FDA can determine about half of the approximately 300 pesticides with EPA tolerances, as well as others that have no tolerances. The most common MRMs can also detect many metabolites, impurities, and alteration products of pesticides with and without tolerances.

Single residue methods (SRMs) or selective MRMs are used to determine pesticides not covered by an MRM. An SRM generally determines 1 pesticide; a selective MRM measures a relatively small number of chemically related pesticides, such as phenylurea herbicides. These types of methods are more resource-intensive per residue, and they may require at least as much time to perform as an MRM.

The lower limit of measurement in FDA's determination of a specific pesticide residue is usually well below tolerance levels, which generally range from 0.1 to 50 parts per million (ppm). Residues present at 0.01 ppm and above are usually measurable; however, for individual pesticides, this limit may range from 0.005 to 1 ppm. In this report, the term "trace" is used to indicate residues detected at levels below the limit of residue measurement.

FDA acquires pesticide residue data from states through Foodcontam.

▽ FDA/State Cooperation

Personnel in FDA field offices interact with their counterparts in states to carry out more effective pesticide residue monitoring. The types and amount of interaction between FDA and individual states vary, but nearly all states have some cooperative effort.

FDA also places value on acquiring and using state-generated pesticide residue data to complement its own residue program. For several years, FDA has supported, through a contract with Mississippi State University (MSU), the "Foodcontam" data base, which is a compilation of state-collected residue data.

▽ Animal Feeds

FDA's Center for Veterinary Medicine (CVM) collects and analyzes domestic and imported feeds for pesticide residues under its Feed Contaminants Compliance Program.

In addition to feed monitoring by FDA personnel, CVM also reviews pesticide residue data supplied by various states under "Feedcon," a data base that is operated by MSU under the auspices of the Association of American Feed Control Officials. CVM periodically reviews these data so that potential pesticide residue problems can be identified.

▽ International Information

FDA has subscribed for a number of years to the Battelle World Agrochemical Data Bank, a computer data base containing pesticide usage data for about 20-25 countries that export food to the United States. These data on fungicides, herbicides, and insecticides for agricultural use have been provided each year to FDA Districts to assist them in planning and conducting pesticide monitoring. A new contract was negotiated in 1991 and was awarded early in 1992 to Landell Mills, Bath, England. Through this contract, FDA anticipates gaining additional information on foreign pesticide usage.

In addition to obtaining foreign pesticide usage information under contract with commercial firms, other steps are being taken by FDA to meet the requirements of the Pesticide Monitoring Improvements Act of 1988. Progress continues in implementing the pesticide monitoring data management provision and analytical methods research plan provision of the Act. FDA continues to

work with foreign governments to obtain information on pesticide usage in these countries and with USDA's National Agricultural Statistics Service to obtain domestic usage data.

Incidence/Level Monitoring

This approach is complementary to regulatory monitoring, and it is an attempt to fill gaps in FDA knowledge about particular pesticide/commodity combinations by analyzing randomly selected samples to determine the presence and levels of certain pesticides. In 1990, surveys of selected aquaculture products, milk, and processed foods were conducted under this monitoring approach. These 3 projects were continued in 1991, and the processed foods survey was expanded to include other commodities.

FDA pesticide residue data collected over the last 30 years are extensive; however, the monitoring program used to collect these data was not designed to produce data that are statistically representative of the overall residue situation for a particular pesticide, commodity, or place of origin. In FDA's surveillance sampling, some bias may be incurred by weighting sampling toward such factors as commodity or place of origin with a past history of violations or large volume of production or import. In addition, the total number of samples of a given commodity analyzed for a particular pesticide each year may not be sufficient to draw specific conclusions about the residue situation for the whole volume of that commodity in commerce.

As part of FDA's continuing effort to improve its pesticide program, statistically based monitoring will be performed to determine how data on violation rates, frequency of occurrence (incidence), and residue levels obtained from such a sampling approach compare with those obtained from FDA's traditional surveillance sampling. Tomatoes and pears have been selected for the initial survey.

The sampling frames have been chosen and FDA statisticians have designed an objective sampling plan that attempts to ensure that any fresh tomatoes and pears moving in commerce in a given time period have a chance of being sampled.

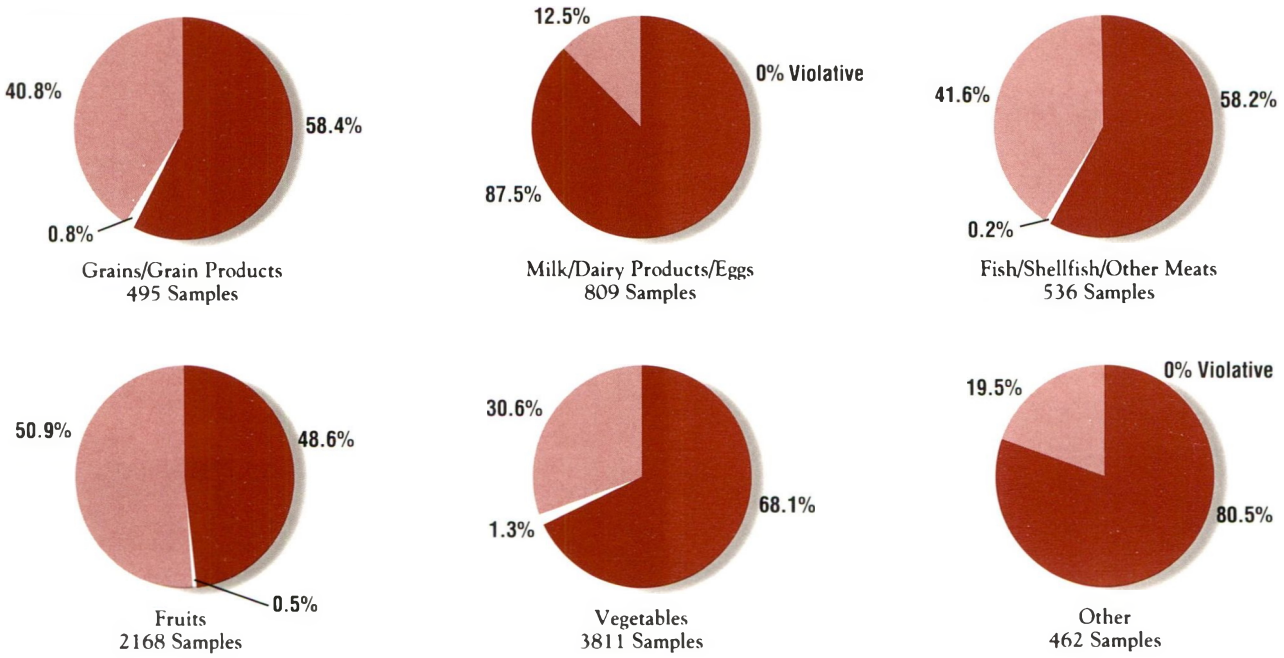
Total Diet Study

The Total Diet Study is another major element of FDA's pesticide monitoring program. This approach is designed to estimate dietary intakes of pesticide residues by 8 age/sex groups ranging from infants to senior citizens. FDA personnel purchase foods from supermarkets or grocery stores 4 times per year, once from each of 4 geographical regions of the country. Each collection contains 234 food items that have been selected on the basis of information obtained through nationwide dietary surveys. The 234 foods are representative of over 5000 different foods in the national surveys; for example, apple pie represents all fruit pies and fruit pastries. Each of the 4 collections is a composite of like foods purchased in 3 cities in that region. The foods are prepared table-ready and then analyzed for pesticide residues (as well as radionuclides, industrial chemicals, toxic metals, and essential minerals). The levels of pesticides found, along with food consumption data, are used to estimate the dietary intakes of the pesticide residues.

The Total Diet Study estimates dietary intakes of pesticides.

FIGURE 1

SUMMARY OF RESULTS (DOMESTIC) BY COMMODITY GROUP OF 1991 SAMPLE ANALYSIS FOR PESTICIDE RESIDUES
(Surveillance Samples Only)



RESULTS AND DISCUSSION

Regulatory Monitoring

In 1991, 19,082 samples (18,214 surveillance and 868 compliance) were analyzed by FDA under regulatory monitoring. Of these, 8466 were domestic and 10,616 were imports, as FDA continues to emphasize testing of imported food shipments.

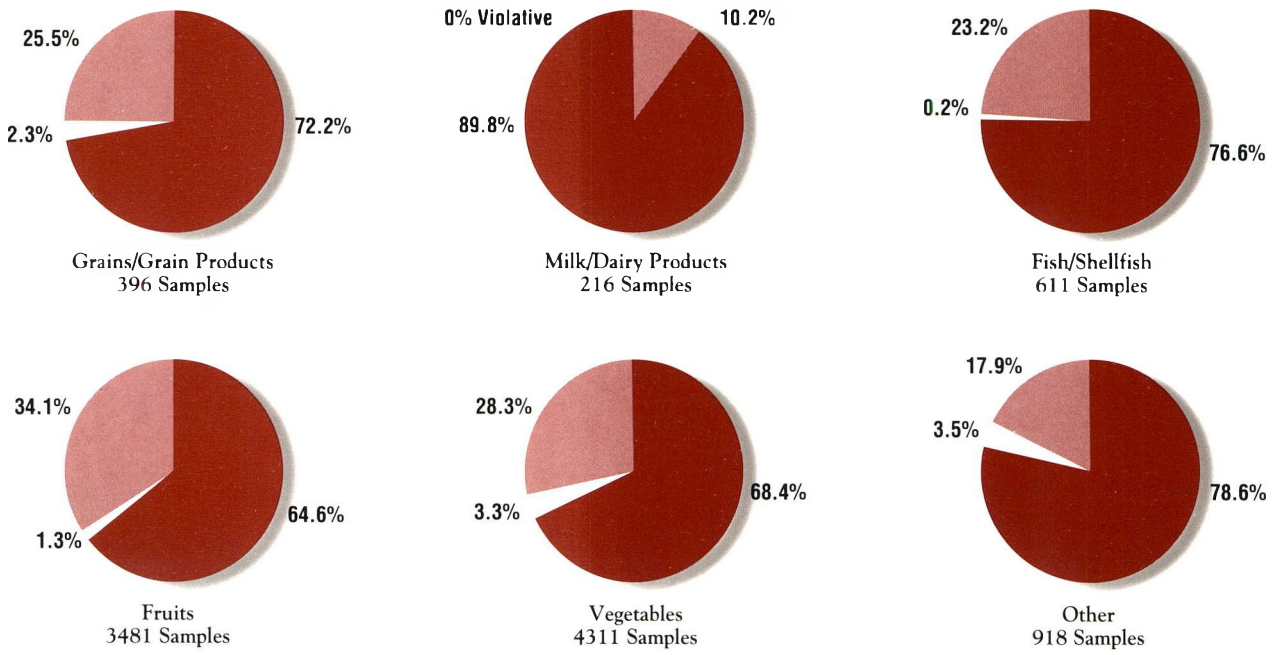
Figure 1 shows the percentage of the 8281 domestic surveillance samples by commodity group with no residues found, nonviolative residues found, and violative residues found. As in previous years, fruits and vegetables comprised the largest proportion of the commodities analyzed in 1991; those 2 commodity groups accounted for 72% of the total number of domestic surveillance samples. In 1991, no violative residues were found in 99% of all domestic surveillance samples.

Appendix A contains more detailed data on domestic surveillance findings by commodity, including the total number of samples analyzed, the percent samples with no residues found, and the percent violative samples. Of the 8281 domestic surveillance samples, 64% had no detectable residues, less than 1% had over-tolerance residues, and less than 1% had residues for which there was no tolerance for that particular pesticide/commodity combination. Fruits and

FIGURE 2

SUMMARY OF RESULTS (IMPORT) BY COMMODITY GROUP OF 1991 SAMPLE ANALYSIS FOR PESTICIDE RESIDUES

(Surveillance Samples Only)



vegetables, the largest commodity groups, had 49 and 68% samples, respectively, with no residues detected. Less than 1% of the fruit samples and less than 2% of the vegetable samples contained violative residues (Figure 1). In the milk/dairy products/eggs group, 88% of the samples had no residues found, and no violative residues were found. In the category Other, which includes a variety of foods, 81% of the samples had no detectable residues, and none had violative residues; this category included 155 baby food samples.

The findings by commodity group for the 9933 import surveillance samples are shown in Figure 2. Fruits and vegetables accounted for 78% of these samples. Overall, no violative residues were found in nearly 98% of the import surveillance samples (97% in 1989 and 96% in 1990).

Appendix B contains detailed data on the import surveillance samples. Of the 9933 samples analyzed, 69% had no residues detected, less than 1% had over-tolerance residues, and 2% had residues for which there was no tolerance for that particular pesticide/commodity combination. Fruits and vegetables had 65 and 68%, respectively, with no residues detected. Each group had less than 1% with over-tolerance residues. One percent of the fruit samples and 3% of the vegetable samples had residues for which there was no tolerance. No residues were found in 90% of the milk/dairy products group and no samples were violative.

▽ Geographic Coverage

Domestic.— In 1991, domestic surveillance samples were collected from all 50 states and Puerto Rico. The largest numbers of samples were collected from the major agricultural states.

Import.— Samples of shipments from 102 countries were collected. Table 1 lists the numbers of samples collected and the countries from which they originated. As usual, Mexico was the source of the highest number of samples, reflecting the volume and variety of commodities imported from that country, especially during the winter months.

▽ Pesticide Coverage

Table 2 lists the 298 pesticides that were detectable in foods analyzed by the methods used; the 108 pesticides actually found are also indicated.

A number of pesticides were added to the detectable list in 1991 because ongoing research demonstrated their recovery through the analytical methods used. Some pesticides that were listed as detectable in previous years are not shown in the 1991 list because they are not of current interest. These pesticides are no longer manufactured/sold, have no tolerances, no known foreign use, and no historical findings in FDA monitoring.

In 1991, about 8% of the 19,082 samples were analyzed using 1 or more SRMs or selective MRMs. Of those samples, 44% were domestic and 56% were import. (About 13% of the compliance samples and 8% of the surveillance samples were analyzed using SRMs or selective MRMs.)

TABLE 1

FOREIGN COUNTRIES AND NUMBER OF SAMPLES[a] COLLECTED AND ANALYZED IN 1991

Country	Number of Samples	Country	Number of Samples
Mexico	2719	Grenada	28
Chile	961	El Salvador	26
The Netherlands	654	Yugoslavia	26
Canada	602	Morocco	25
New Zealand	552	Pakistan	25
Italy	505	Panama	23
France	346	Egypt	17
Costa Rica	304	Guyana	16
Guatemala	269	Lebanon	16
Dominican Republic	236	Singapore	15
Ecuador	236	Poland	14
Thailand	219	Switzerland	14
Spain	198	Ireland	13
India	181	Bahamas	11
China, People's Rep. of	153	Sweden	11
Honduras	143	Unspecified	24
Colombia	139		
Taiwan	134	**Ten or fewer samples collected from the following:**	
Belgium	121	Bangladesh	Nicaragua
Jamaica	115	Belize	Nigeria
Australia	114	Bolivia	North Korea
Korea, Rep. of	112	Bulgaria	Norway
Argentina	107	Burma	Papua New Guinea
Greece	97	Cambodia	Romania
Japan	90	Cameroon	Saudi Arabia
Trinidad & Tobago	88	Cyprus	South Africa
Israel	85	Czechoslovakia	Soviet Union
Philippines	79	Dominica	Sri Lanka
Germany	74	Ethiopia	St. Lucia
Haiti	55	Finland	St. Vincent
Brazil	52	French Guiana	Sudan
Turkey	52	Ghana	Surinam
United Kingdom	52	Iceland	Syria
Hong Kong	51	Ivory Coast	Tanzania
Denmark	48	Jordan	Tonga
Indonesia	33	Kenya	Uganda
Peru	33	Macau	Uruguay
Austria	32	Madagascar	Western Samoa
Venezuela	32	Malawi	Yemen
Hungary	30	Malaysia	
Portugal	30	Mozambique	
Fiji	28	Netherlands Antilles	

[a] Surveillance plus compliance samples.

▽ Targeted Pesticides

FDA conducts a number of surveys each year to obtain information on the occurrence of a particular pesticide or group of chemically related pesticides in several different commodities. These surveys may be targeted toward pesticide/food combinations not usually covered by regulatory monitoring or may be performed to gain information on pesticides of special interest. Table 3 lists a number of the targeted combinations covered in 1991. Additional information is given below for some of these combinations.

TABLE 2

PESTICIDES DETECTABLE BY THE METHODS USED AND PESTICIDES FOUND (*) IN 1991 REGULATORY MONITORING[a]

Acephate*
Acetochlor
Alachlor
Aldicarb*
Aldoxycarb*
Aldrin*
Allethrin*
Allidochlor
Alpha-cypermethrin
Ametryn
Aminocarb
Amitraz*
Anilazine*
Aramite
Atrazine
Azinphos-ethyl
Azinphos-methyl*
Bendiocarb
Benfluralin
Benomyl*
Bensulide
Benzoylprop-ethyl
BHC*
Bifenox
Bifenthrin
Binapacryl
Bromacil
Bromophos
Bromophos-ethy
Bromopropylate
Bromoxynil
Bufencarb
Butachlor
Butocarboxim
Butralin
Captafol*
Captan*
Carbaryl*
Carbendazim*
Carbofuran*
Carbon tetrachloride
Carbophenothion*
Carbosulfan
Carboxin
Chlorbenside
Chlorbromuron
Chlordane*
Chlordecone
Chlorfenvinphos*
Chlorflurecol
Chlornitrofen
Chlorobenzilate
Chloroform
3-Chloro-5-methyl-4-nitro-1H-pyrazole (Release)
Chloroneb
Chloropropylate
Chlorothalonil*
Chloroxuron
Chlorpropham*
Chlorpyrifos*
Chlorpyrifos-methyl*
Chlorthiophos
Clomazone
Coumaphos
Crotoxyphos
Crufomate
Cyanazine
Cyanofenphos
Cyanophos
Cycloate
Cyfluthrin
Cyhalothrin, lambda
Cyhexatin*
Cypermethrin*
Cyprazine
2,4-D
Daminozide*
DCPA*
DDT*
DEF
Demeton*
Dialifor
Di-allate
Diazinon*
Dichlobenil*
Dichlofenthion
Dichlofluanid*
Dichlone
Dichlorvos*
Diclobutrazol
Diclofop-methyl
Dicloran*
Dicofol*
Dicrotophos
Dieldrin*
Diethatyl-ethyl
Dimethametryn
Dimethoate*
Dinitramine
Dinocap
Dioxabenzofos
Dioxacarb
Dioxathion
Diphenylamine*
Diquat
Disulfoton*
Diuron
Endosulfan*
Endrin*
EPN*
EPTC
Esfenvalerate*
Etaconazole
Ethalfluralin
Ethiofencarb
Ethiolate
Ethion*
Ethoprop
Ethoxyquin
Ethylenebisdithio-carbamates*[b]
Ethylene dibromide*
Ethylene dichloride
Etridiazole
Etrimfos
Famphur
Fenamiphos
Fenarimol*
Fenfuram
Fenitrothion*
Fenobucarb
Fenoxaprop ethyl ester
Fenoxycarb
Fenpropathrin
Fenson
Fensulfothion
Fenthion*
Fenvalerate*
Flamprop-M-isopropyl
Flamprop-methyl
Fluazifop butyl ester
Fluchloralin
Flucythrinate
Flusilazole
Fluvalinate*
Folpet*
Fonofos*
Formetanate hydrochloride*
Formothion
Gardona
Heptachlor*
Heptenophos
Hexachlorobenzene*
Hexazinone
Imazalil*
Imazamethabenz-methyl
Iprobenfos
Iprodione*
Isazofos
Isofenphos
Isoprocarb
Isopropalin
Lactofen
Leptophos
Lindane*
Linuron*
Malathion*
Mecarbam*
Mephosfolan
Merphos
Metalaxyl
Metasystox thiol
Metazachlor
Methamidophos*
Methidathion*
Methiocarb*
Methomyl*
Methoxychlor*
Metobromuron
Metolachlor
Metolcarb
Metribuzin
Mevinphos*
Mirex
Monocrotophos*
Monolinuron
Myclobutanil*
Naled
Neburon
Nitralin
Nitrapyrin
Nitrofen
Nitrofluorfen
Nitrothal-isopropyl
Norflurazon
Nuarimol
Octhilinone
Ofurace
Omethoate*
Ovex
Oxadiazon*
Oxamyl*
Oxydemeton-methyl*
Oxyfluorfen
Paclobutrazol
Paraquat*
Parathion*
Parathion-methyl*
Penconazole
Permethrin*
Perthane
Phenothrin
Phenthoate
Phenylphenol, ortho*
Phorate*
Phosalone*
Phosmet*
Phosphamidon*
Phosphine*[c]
Phoxim
Picloram
Piperophos
Pirimicarb
Pirimiphos-ethyl
Pirimiphos-methyl*
Primisulfuron-methyl
Probenazole
Prochloraz
Procyazine
Procymidone*
Profenofos*
Profluralin
Promecarb
Prometryn
Pronamide*
Propachlor
Propanil
Propargite*
Propazine
Propetamphos
Propham
Propiconazole
Propoxur*
Prothiofos*
Prothoate
Pyrazon
Pyrazophos*
Pyrethrins
Pyridaphenthion
Quinalphos*
Quintozene*
Quizalofop ethyl ester
Ronnel*
Schradan
Simazine
Simetryn
Strobane
Sulfallate
Sulfotep*
Sulfur*
Sulfur dioxide*
Sulphenone
Sulprofos
TDE*
Tecnazene*
Terbacil
Terbufos
Terbuthylazine
Terbutryn
Tetradifon*
Tetraiodoethylene
Tetrasul
Thiabendazole*
Thiobencarb
Thiodicarb
Thiometon
Thionazin
Thiophanate-methyl
Tolylfluanid*
Toxaphene*
Tralomethrin
Triadimefon*
Triadimenol
Tri-allate
Triasulfuron
Triazophos
Trichlorfon*
Trichloronat
Triclopyr
Tricyclazole
Tridiphane
Triflumizole
Trifluralin*
Triforine
Trimethacarb
Triphenyltin hydroxide*
Vernolate
Vinclozolin*
XMC

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

[b] Such as maneb.

[c] Such as aluminum phosphide.

TABLE 3

PESTICIDE/COMMODITY/LOCATION TARGETED MONITORING CONDUCTED IN 1991

Pesticide	Commodity	Country
Aldicarb	fruits, root/tuber vegetables	various
	lemons	Chile
Amitraz	milk	USA
Atrazine	corn	USA
Benomyl	fruits, leaf/stem vegetables, peppers	various
Bromoxynil	wheat	USA
Carbophenothion	soybeans	USA
Daminozide/UDMH	apples, cherries	Canada, USA
Dinocap	grapes	Chile
EBDCs[a]	coffee beans, leaf/stem vegetables, peppers, snow/sugar snap peas	various
Formothion	olives	Spain
N-Methylcarbamates, organochlorine, organophosphorus	pineapples	Honduras
Paraquat	asparagus, potatoes	USA
Permethrin	pumpkins	USA
Procymidone	wine	various
Propargite	apples, cranberries	USA
Triphenyltin hydroxide	pecans	USA

[a] Such as maneb.

Aldicarb. In 1991, a total of 2575 samples (1045 domestic and 1530 import) were analyzed for total aldicarb (includes aldicarb, aldoxycarb, and aldicarb sulfoxide). (Many of these analyses would also have detected other carbamate residues.) Included in the total number of samples were the following: tropical fruits, 527; root/tuber vegetables, 326; vine fruits, 203; peppers, 177; berries, 174; baby foods containing bananas, 145; citrus fruits and juices, 134; and pit fruits, 131.

Of the 527 tropical fruit samples analyzed for total aldicarb, 398 were bananas, which were analyzed primarily in response to concern about atypically high levels of this pesticide found in some field samples of bananas by the aldicarb registrant. For the initial analysis, 6 bananas from an 18-lb (8.2-kg) sample were halved lengthwise, composited (including the skin), and analyzed. If the analytical result for the 6-banana composite was equal to or >0.05 ppm aldicarb, indicating that an individual banana could have contained the tolerance level of 0.3 ppm aldicarb, then the 6 remaining banana halves were individually analyzed.

Thirty-one of the 398 6-banana composites had detectable aldicarb residues ranging from 0.01 to 0.15 ppm. Twelve of the 31 composites had aldicarb residue levels equal to or >0.05 ppm and, thus, had individual banana analyses performed. These results ranged from 0.01 to 0.40 ppm aldicarb. Two of these residues were >0.3 ppm aldicarb. Of the 145 banana-containing baby food samples, 2 had 0.01 ppm aldicarb.

Although FDA and industry monitoring of bananas and banana products for aldicarb did not demonstrate frequent findings of aldicarb, the banana industry elected to voluntarily discontinue its use. Of all the remaining food samples analyzed for aldicarb, only 1, a domestic potato sample, contained 0.01 ppm aldicarb (the tolerance is 1 ppm). Residues at or above 0.01 ppm would have been determined.

Benomyl. A total of 1286 samples (236 domestic and 1050 import) were analyzed for benomyl and related compounds (determined as their common alteration product carbendazim). Emphasis was placed on leaf/stem vegetables, 210; berries (including grapes), 121; peppers, 116; pit fruits, 110; tropical fruits, 104; and vine fruits, 103.

Residues were found in 35 samples (peas, 8; cherries, 7; pears, 4; nectarines, 3; bananas, cantaloupes, peaches, and plums, 2 each; and cucumbers, green beans, oranges, raspberries, and watermelons, 1 each) at levels ranging from 0.05 ppm in bananas to 2.4 ppm in peas. The tolerances for the above fruits and vegetables range from 0.2 ppm for bananas (without peel) to 15 ppm for cherries, nectarines, peaches, and plums. There is no tolerance for benomyl in/on peas; therefore, the 8 samples of peas were violative. Residues at or above 0.05 ppm would have been determined.

Ethylenebisdithiocarbamates/Ethylenethiourea (EBDCs/ETU). In 1991, 724 samples (220 domestic and 504 import) were analyzed for residues of EBDCs and/or ETU (a metabolite or by-product of EBDC pesticides). The commodities targeted were leaf/stem vegetables, 153; peppers, 117; snow/sugar snap peas, 79; and coffee beans, 60.

Residues were found in 110 samples, nearly half of them imported snow/sugar snap peas. Another one-third of the residues were found in the leaf/stem

vegetables group, especially in collard and mustard greens. Six samples, all snow/sugar snap peas, were violative, with EBDC levels ranging from 7.8 to 22.4 ppm. The tolerance for peas is 7 ppm. Residues of EBDCs at about 0.1 ppm or above would have been determined.

A special survey of green coffee beans was performed by FDA's San Francisco District. Sixty green coffee samples were collected from shipments from 21 different African, Asian, and Central and South American countries that are the major exporters of coffee beans to the United States The samples were analyzed in the San Francisco District Laboratory for EBDCs. No EBDC residues were found. The samples were also analyzed for benomyl, *N*-methyl-carbamate, and organochlorine/organophosphorus residues. Three samples had residues of chlorpyrifos at 0.01, 0.02, and 0.04 ppm, respectively, and 1 sample had a residue of pirimiphos-methyl at 0.01 ppm.

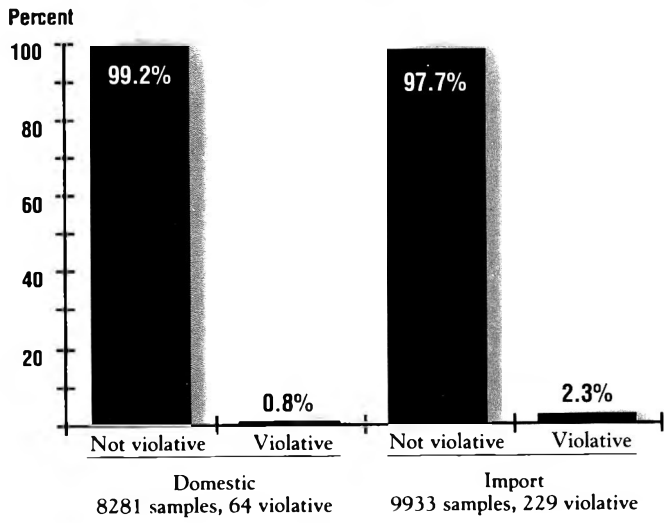
Procymidone. On April 26, 1991, EPA set a time-limited tolerance of 7 ppm for residues of procymidone in wine grapes grown before January 1, 1990. The tolerance also covers procymidone residues in wine. The tolerance, which expires on April 26, 1995, applies to wines produced from grapes harvested before January 1990 (northern hemisphere) or March 1990 (southern hemisphere).

FDA continued to monitor wine for procymidone residues in 1991, as it had in 1990. In 1991, a total of 501 import wine samples were analyzed using a method that can determine procymidone at levels of 0.02 ppm and above. Of the 501 samples, 51 were compliance and 450 were surveillance. Of the compliance samples, 37% had no detectable procymidone residues, 12% had violative residues, and the highest residue was 0.06 ppm. The 450 surveillance samples included 75% with no detectable procymidone residues and 3% with violative residues up to 0.10 ppm. Wines in which violative procymidone residues

FDA targeted a number of pesticides in 1991.

FIGURE 3

DOMESTIC AND IMPORT SURVEILLANCE SAMPLE VIOLATION RATES FOR 1991



were found were detained and subsequently released after the time-limited tolerance was established.

Since the regulation on procymidone in wines was promulgated, FDA has found no wines with illegal procymidone residues.

▽ Surveillance/Compliance Violation Rate Comparison

As shown in Figure 3, 0.8% of the domestic surveillance samples and 2.3% of the import surveillance samples were violative. Surveillance sampling may be based on a number of factors, including usage patterns and volume, commodity treatment history, dietary importance of the commodity, toxicity of the pesticide, known problem areas, past monitoring results, and local pesticide usage.

TABLE 4

COMPLIANCE SAMPLES BY COMMODITY GROUP IN 1991

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, %
Domestic			
Grains and grain products	3	0	0
Milk/dairy products/eggs	3	100	0
Fish/shellfish/other meats	36	56	0
Fruits	24	46	0
Vegetables	96	36	36
Other	23	83	0
Total	**185**	**48**	**19**
Import			
Grains and grain products	71	66	3
Milk/dairy products	4	100	0
Fish/shellfish	29	55	3
Fruits	216	46	11
Vegetables	232	50	16
Other	131	60	7
Total	**683**	**53**	**11**

In 1991, 185 domestic and 683 import compliance samples were analyzed (Table 4). Because compliance samples are collected when a pesticide residue problem is known or suspected, violation rates are much higher than those for surveillance samples: 19% for domestic (30% in 1990) and 11% for imports (15% in 1990). In the domestic category, all of the violative samples were in the Vegetables group.

Most of the 1991 compliance samples were collected as follow-up on violative surveillance samples. These included follow-up samples from the same shipment as the violative surveillance sample, follow-up samples from additional product from the same grower or shipper, and audit samples from shipments presented for entry into the United States with a certificate of analysis (i.e., shipments subject to automatic detention).

▽ Foodcontam Data

In 1991, 20 states contributed pesticide residue data to Foodcontam, representing 18,928 samples of a wide variety of foods. Table 5 lists the 20 states, the number of samples analyzed by each, and the number and percent of samples with positive and "significant" findings. In this context, a significant finding denotes either a residue that exceeds federal or state regulatory limits or a residue for which there is no tolerance for the particular chemical/food product, or it reflects some other unusual finding(s). Of the 18,928 samples analyzed, the residue findings for <1% were categorized as significant, which is in general agreement with FDA's monitoring results.

▽ Animal Feeds

In 1991, 601 domestic feed samples (593 surveillance and 8 compliance) and 74

import feed samples (49 surveillance and 25 compliance) were collected by FDA field offices. Of the 593 domestic surveillance samples, 190 (32%) contained no detectable pesticide residues, and 12 (2%) contained violative pesticide residues (Table 6). Of the 49 import surveillance samples, 22 (45%) contained no detectable residues, and 1 sample (2%) contained violative residues. The 13 violative surveillance samples were 3 feeds with residues at levels that exceeded the tolerance or action level and 10 feeds with residues for which there was no tolerance. Regulatory actions were initiated at the FDA District Level when violative residues were found.

In the 403 domestic surveillance feed samples in which pesticide residues were detected, malathion, chlorpyrifos-methyl, diazinon, and chlorpyrifos were the most frequently found residues and accounted for 74% of all pesticide residues detected. The levels at which they were found were as follows: malathion, trace–4.1 ppm; chlorpyrifos-methyl, trace–3.8 ppm; diazinon, trace–0.7 ppm; and chlorpyrifos, trace–0.5 ppm.

Summary: Regulatory Monitoring

In 1991, no residues were found in 64% of the domestic surveillance samples or 69% of the import surveillance samples (Figure 4) that were collected and analyzed under regulatory monitoring. Less than 1% of the domestic and import surveillance samples had levels of residues that were over tolerance. Less than 1% of the domestic and 2% of the import surveillance samples had residues for which there was no tolerance. These findings are similar to those from previous years of FDA regulatory monitoring.

Incidence/Level Monitoring

In 1991, 3 projects were performed under this approach to monitoring.

▽ Aquaculture Survey

Aquaculture, which is the breeding, rearing, and harvesting of fish/shellfish under environmentally controlled conditions, began over 100 years ago in the United States. The industry has expanded in recent years to meet increasing consumer consumption of fish and shellfish. In 1989, 12% of U.S. fish consumption came from aquaculture, 4 times the level 10 years ago.

In view of the continuing growth of the aquaculture industry and increasing consumption of seafood, the survey of

TABLE 5

SUMMARY OF FOODCONTAM FINDINGS FOR 1991

State	Total Samples	No. Positive	Positive, %	No. Significant[a]	Significant,[a] %
Arkansas	187	27	14.4	0	0
California	6667	1354	20.3	58	0.9
Connecticut	369	205	55.6	6	1.6
Florida	3567	1018	28.5	22	0.6
Georgia	566	149	26.3	14	2.5
Hawaii	274	168	61.3	5	1.8
Indiana	592	532	89.9	0	0
Maine	14	4	28.6	0	0
Massachusetts	2	0	0	0	0
Michigan	534	171	32.0	0	0
Minnesota	175	28	16.0	0	0
New York	1466	321	21.9	7	0.5
North Carolina	557	127	22.8	2	0.4
Oregon	533	73	13.7	7	1.3
Pennsylvania	389	205	52.7	7	1.8
Rhode Island	83	10	12.0	6	7.2
Virginia	1267	301	23.8	42	3.3
Washington	655	65	9.9	0	0
Wisconsin	942	14	1.5	3	0.3
Wyoming	89	0	0	0	0
Total	**18,928**	**4772**	**25.2**	**179**	**0.9**

[a] Significant finding denotes either a residue that exceeds federal or state regulatory limits or a residue for which there is no tolerance for the chemical/food combination, or it reflects an unusual finding.

selected aquaculture products begun in 1990 was continued in 1991. This survey focused on persistent halogenated pesticides, which may be present as a result of past agricultural uses.

Seven FDA Districts collected a total of 188 samples (this number is included in the count under Fish & shellfish in Appendix A) from important domestic aquaculture areas and shipped them to the Buffalo District Laboratory for analysis. The samples included 128 catfish, 19 crawfish, 19 trout, 6 shrimp, 5 oysters, and 11 other types. These species were selected because they represent major aquaculture products.

Sixty-five of the samples had no detectable pesticide residues, 68 had levels of <0.01 ppm of 1 or more residues, and 55 had quantifiable levels of 1 or more residues. One of 5 catfish samples collected by one FDA District contained DCPA (an herbicide, trade name Dacthal) at 0.07 ppm. One of 17 catfish samples collected by another FDA District was found to contain 0.04 ppm chlorpyrifos. Tolerances have not been established for either of these chemicals in fish; therefore, the collecting Districts were notified of these findings to allow them to follow up on the incidents.

The other findings were the following:

Pesticide	No. of Samples with Residues	Amt Found, ppm	FDA Action Level, ppm
DDT, total	119	<0.01–0.27	5
Dieldrin	31	<0.01–0.02	0.3
BHC	21	<0.01	–
Chlordane, total	3	<0.01–0.02	0.3

No pesticide residues were found that exceeded EPA tolerances or FDA action levels. (In this context, an action level is a guideline, i.e., a legally nonbinding FDA regulatory limit for a pesticide that is no longer permitted for food use but may persist in the environment and, thus, may unavoidably contaminate food. Chlorinated pesticides such as those listed above are a class of chemicals for which action levels have been set.)

TABLE 6

NUMBERS OF SAMPLES AND VIOLATION RATES IN DOMESTIC SURVEILLANCE FEED SAMPLES IN 1991

Type of Feed	Total No. of Samples	Samples with No Residues Found		Violative Samples	
		No.	%	No.	%
Mixed feed ration	216	59	27	–	–
Animal by-products	121	52	43	–	–
Whole/ground grains	112	32	29	1	<1
Plant by-products	111	27	24	7	6
Hay	24	12	50	4	17
Minerals	9	8	89	–	–
Total	**593**	**190**	**32**	**12**	**2**

Milk Survey

EPA operates the Environmental Radiation Ambient Monitoring System (ERAMS), which collects and composites samples of pasteurized whole milk from metropolitan areas throughout the United States for the purpose of monitoring radionuclides in milk. These composites are also analyzed for pesticide residues through an FDA-supported contract.

From May 1990 through July 1991, 806 milk samples from 63 metropolitan areas

were collected and analyzed. (This project is not operated on a Fiscal Year basis.) Milk samples from 8 of the metropolitan areas had no detectable residues for the 15-month period. Pesticide residues were found in 398 (49.4%) of the samples. The most frequently found residues were *p,p'*-DDE (212 findings) and dieldrin (172). The highest residue level measured was 0.02 ppm *p,p'*-DDE (whole milk basis). These chlorinated pesticides have not been registered for agricultural use for about 20 years; however, because of their persistence in the environment, they still are present at low levels in some foods, especially those of animal origin.

▼ Processed and Other Foods Survey

In 1991, FDA continued its cooperative agreement with the USDA Animal and Plant Health Inspection Service's (APHIS) National Monitoring and Residue Analysis Laboratory (NMRAL) located in Gulfport, MS, whereby NMRAL would perform FDA-directed pesticide residue analysis. FDA entered into this agreement to generate monitoring data on specific, important commodity/chemical combinations to complement FDA's regulatory-oriented pesticide approach.

In 1991, NMRAL completed 3 assignments begun in 1990 and 3 new assignments. The 1990 assignments completed in 1991 were daminozide in processed foods, ETU in processed foods, and pesticide residues (daminozide, ETU, *N*-methylcarbamates, organochlorine, and organophosphorus; in 1991, benomyl and thiabendazole were added) in commercially prepared baby foods. The 3 new assignments were aldicarb (and other *N*-methylcarbamates) in bananas and citrus fruits, benomyl (and thiabendazole) in foods with postharvest and food additive tolerances, and methyl bromide in dried beans, dried fruit, and rice.

For the assignments undertaken in 1990 and the aldicarb in bananas and citrus fruits assignment, samples were obtained through FDA contracts with state government agencies in Illinois, Massachusetts, Michigan, Minnesota, Washington, and Wisconsin. NMRAL personnel also collected samples amounting to one-quarter of the total. The sampling was designed to provide broad geographical coverage (East Coast, North Central, West Coast, southern United States). For the 1991 benomyl and methyl bromide assignments, new FDA contracts were signed with state government agencies in Massachusetts, Minnesota, New York, and Washington.

FIGURE 4

COMPARISON OF RESULTS FOR DOMESTIC AND IMPORT SURVEILLANCE SAMPLES IN 1991

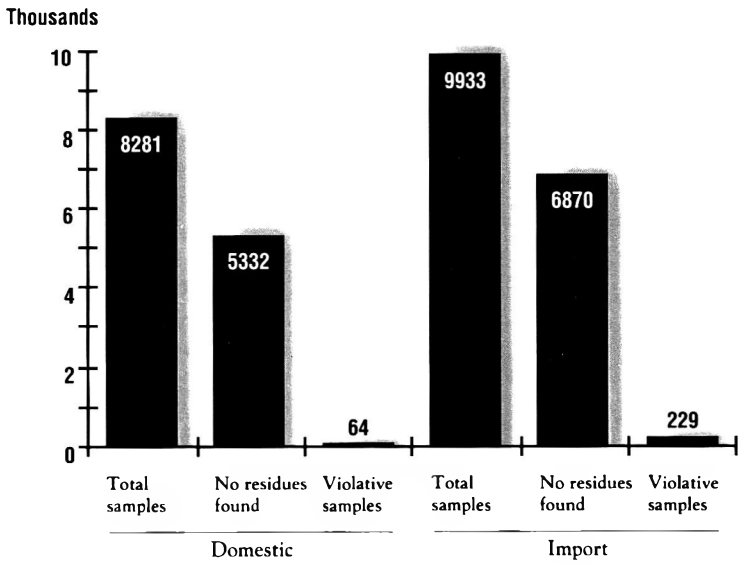


TABLE 7

COMMODITIES/PESTICIDES ANALYZED UNDER APHIS/NMRAL ASSIGNMENTS IN 1991

Commodity	Pesticide Residue	No. of Analyses
Baby foods	benomyl (and thiabendazole)	822
	daminozide	401
	ETU	205
	N-methylcarbamates	697
	organochlorine and organophosphorus	179
Bananas and citrus fruits	aldicarb (and other *N*-methylcarbamates)	453[a]
Dried beans, dried fruit, and rice	methyl bromide	628
Foods with postharvest uses and/or food additive tolerances	benomyl (and thiabendazole)	1018
Processed foods	daminozide	460
Processed foods	ETU	702
	Total	**5565**

[a] The banana-containing baby foods analyzed under this assignment are counted under baby foods.

Incidence/level monitoring findings were consistent with those from regulatory monitoring.

Nine analytical methods, applicable to about 35 separate method/commodity combinations, were validated for the assignments.

Table 7 shows the commodities/pesticides covered and the numbers of analyses performed under the various task assignments. A total of 600 occurrences of residues were found in the 5565 analyses performed. In some cases, multiple residues were reported in a single sample. No residues were found that were over tolerance or for which a tolerance did not exist. (Violative findings would have been followed up on a regulatory basis by FDA if appropriate.)

Baby Foods. There were 138 occurrences of pesticide residues in baby foods analyzed in 1991. Residues of 8 pesticides were found: aldicarb, trace; benomyl, trace–0.27 ppm; carbaryl, trace–0.15 ppm; chlorpyrifos, 0.02 ppm; daminozide, trace; ETU, trace–0.02 ppm; malathion, trace–0.40 ppm; and thiabendazole, trace–0.26 ppm.

Bananas and Citrus Fruits. Aldicarb residues were found in 13 of 176 banana samples at levels of trace–0.19 ppm.

Dried Beans, Dried Fruit, and Rice. Methyl bromide residues were found in 1 of 22 samples of black-eyed peas at 0.04 ppm and in 1 of 161 samples of raisins at 0.03 ppm.

Foods With Postharvest Uses and/or Food Additive Tolerances. Overall, benomyl residues (determined as carbendazim) were found in 121 samples at levels of trace–7.5 ppm. Thiabendazole was found in 281 samples

at levels of trace–5.6 ppm. The commodities and the amounts of benomyl/thiabendazole found are summarized below.

Commodity	No. of Samples Analyzed/No. of Samples with Residues	Amount Residue Found, ppm
Apples	152/3	trace –0.07 benomyl
	152/117	trace –4.2 thiabendazole
Bananas	64/24	trace –0.26 thiabendazole
Citrus fruits	105/80	trace –5.6 thiabendazole
Mushrooms, fresh	90/25	0.06 – 0.84 benomyl
	90/40	trace –2.7 thiabendazole
Pears	49/4	trace –0.33 benomyl
	49/17	trace –1.2 thiabendazole
Pineapple	62/10	trace –7.5 benomyl
Pit fruit	247/79	trace –1.9 benomyl
	247/1	0.06 thiabendazole
Raisins	110/1	trace thiabendazole
Tomatoes, processed	139/1	trace thiabendazole

Processed Foods. Daminozide was found in 15 food samples: apple products (6 samples) at trace–0.05 ppm, peanut butter (5 samples) at trace–0.11 ppm, and peanuts (4 samples) at trace–0.84 ppm.

ETU was found in 62 samples: grape products (9 samples) at trace–0.02 ppm, potatoes (28 samples) at trace–0.02 ppm, spinach (18 samples) at trace–0.51 ppm, and tomatoes (7 samples) at trace–0.02 ppm.

Summary: Incidence/Level Monitoring

The results of 3 surveys performed under this approach, which included analyses of 188 samples of aquaculture products and 806 samples of milk and 5565 analyses performed on a variety of dried, fresh, and processed foods, were consistent with FDA's regulatory findings. All residues found were below EPA tolerances or FDA action levels except for 2 findings in catfish of chemicals for which there is no tolerance in fish.

Total Diet Study

The Total Diet Study is unique in that it determines pesticide residues in foods that have been prepared as they would be consumed. Of the more than 200 chemicals that can be determined by the analytical methods used, 51 were found in the foods analyzed in 1991, the same number as in 1990. To measure the low levels of pesticides found in the Total Diet Study foods, the analytical methods used are modified to permit measure-

TABLE 8

FREQUENCY OF OCCURRENCE OF PESTICIDES IN TOTAL DIET STUDY IN 1991

Pesticide[a]	Total No. of Findings[b]	Occurrence, %	Pesticide[a]	Total No. of Findings[b]	Occurrence, %
Malathion	167	18	Ethion	21	2
Chlorpyrifos-methyl	97	10	Hexachlorobenzene	20	2
DDT	93	10	Permethrin	16	2
Dieldrin	73	8	BHC, alpha	13	1
Endosulfan	67	7	Chlordane	12	1
Methamidophos	58	6	Parathion	12	1
Chlorpyrifos	51	5	Quintozene	12	1
Dicloran	44	5	Dicofol	10	1
Acephate	42	4			
Diazinon	42	4			
Carbaryl[c]	34	4			
Dimethoate	34	4			
Thiabendazole[d]	31	3			
Chlorpropham	28	3			
Heptachlor	24	3			
Propargite[d]	24	3			
Lindane	22	2			
Omethoate	22	2			

[a] Isomers, metabolites, and related compounds have not been listed separately; they are covered under the generic or "parent" pesticide from which they arise.

[b] On the basis of 936 items.

[c] Reflects overall incidence; however, only 72 selected foods per market basket (i.e., 288 total items) were analyzed for *N*-methylcarbamates.

[d] Reflects overall incidence; however, only 50 selected foods per market basket (i.e., 200 total items) were analyzed for these sulfur-containing compounds.

ment at levels 5–10 times lower than those used in regulatory monitoring. In general, residues present at or above 1 part per billion can be measured. Table 8 lists the 26 most frequently found residues, together with the total number of findings and the percent occurrence in the 936 food items. Malathion continues to be the residue most frequently found because it is used on a wide variety of crops, including many postharvest uses on grains. However, the occurrence has been declining over the past 5 years - from 23 to 18%. DDT has also decreased - from 22 to 10% over the 5-year period.

Pesticide intakes estimated via the Total Diet Study were well below established standards.

Information obtained through the Total Diet Study is used to estimate dietary intakes of pesticides; these intakes are then compared with established standards. One set of standards with which comparisons are made is the ADIs set by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR), panels of experts convened by the United Nations' Food and Agriculture Organization (FAO) and the World Health Organization (WHO). An ADI is the daily intake of a chemical that, if ingested over a lifetime, appears to be without appreciable risk. EPA also sets standards, RfDs, which are estimates of total pesticide exposure to the human population (including sensitive subgroups) that are likely to be without appreciable risk of deleterious effects during a lifetime. The assumptions used by the JMPR in arriving at ADIs and by EPA in setting RfDs differ somewhat; therefore, the standard values may not be the same. Both ADIs and RfDs are revised as new scientific infonnation becomes available. Appendix C lists the ADIs, the RfDs, and the corresponding intakes of pesticide residues by 3 of the 8 age/sex groups covered by the Total Diet Study. As in earlier years, the intakes are well below the standards set by the JMPR and by EPA.

In 1991, the dietary intakes of pesticide residues, as estimated from the Total Diet Study, were well below established standards. These findings are consistent with those from earlier years, as shown in other FDA reports.

Under regulatory monitoring, 19,082 samples of domestically produced food from 50 states and Puerto Rico and imported food from 102 countries were analyzed for pesticide residues in 1991. Of these, 18,214 were surveillance samples, which are collected when there is no suspicion of a pesticide problem. No residues were found in 64% of the domestic or 69% of the import surveillance samples. Findings in the 868 compliance samples reflect that they are collected and analyzed when a pesticide problem is suspected. Under incidence/level monitoring, 188 samples of aquaculture fish/shellfish and 806 samples of whole milk were analyzed for pesticide residues. In addition, 5565 analyses were performed on a variety of dried, fresh, and processed foods. The findings were similar to FDA's regulatory monitoring results. The 1991 Total Diet Study results show that intakes of pesticide residues are generally well below the standards set by the JMPR and by EPA. The overall findings for 1991 are similar to those obtained in previous years.

TABLE 9

FIVE-YEAR (1987-1991) SUMMARY OF NUMBERS OF SAMPLES ANALYZED, PERCENTAGE OF SAMPLES WITH NO RESIDUES FOUND, AND VIOLATION RATES FOR DOMESTIC AND IMPORT SAMPLES[a]

Year	No. of Samples	Domestic			Import		
		Samples with No Residue Found, %	Violative Samples, %		Samples with No Residue Found, %	Violative Samples, %	
			Over Tolerance	No Tolerance		Over Tolerance	No Tolerance
1987	14,492	58	1	1	56	<1	5
1988	18,114	60	<1	1	62	<1	5
1989	18,113	65	<1	<1	67	<1	3
1990	19,146	60	<1	<1	64	<1	4
1991	18,214	64	<1	<1	69	<1	2

[a] Numbers of samples and violation rates for 1987 and 1988 are for both surveillance and compliance samples. For 1989, 1990, and 1991, they are for surveillance samples only.

Table 9 summarizes information on numbers of samples analyzed and violation rates for domestic and import samples for the 5 years covered by FDA's annual reports, 1987–1991. Over this period, the percent of domestic samples with no residues found has ranged from 58 to 65 and for import samples, from 56 to 69. The percent of samples with over-tolerance residues has remained less than 1 for both domestic and imported foods. The percent of no-tolerance residues for domestic samples has been less than 1, and for imports it has ranged from 2 to 5. These 5-year data show the consistently low numbers of violative samples and, thus, confirm the safety of the food supply relative to pesticide residues.

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APPENDIX A

Analysis of Domestic Surveillance Samples by Commodity Group in 1991

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, % Over Tolerance	Samples Violative, % No Tolerance
A. Grains and Grain Products				
Barley	24	33	0	0
Corn, popcorn	84	71	0	0
Oats	48	44	0	2
Rice	15	60	7	0
Soybeans	70	87	0	1
Wheat	176	36	<1	0
Other whole grains	2	50	0	0
Cereal products	17	94	0	0
Milled corn products	13	100	0	0
Rice products	29	100	0	0
Other grain products	17	47	0	0
Total	**495**	**58**	**<1**	**<1**
B. Milk/Dairy Products/Eggs				
Cheese/cheese products	133	97	0	0
Eggs/egg products	236	97	0	0
Milk & cream/milk products	437	80	0	0
Other dairy products	3	33	0	0
Total	**809**	**88**	**0**	**0**
C. Fish/Shellfish/Other Meats				
Fish & shellfish	531	59	0	<1
Other meats	5	0	0	0
Total	**536**	**58**	**0**	**<1**
D. Fruits				
Blackberries	10	70	0	0
Blueberries	44	43	0	0
Cranberries	54	13	0	0
Grapes	231	63	0	0
Raspberries	31	45	0	3
Strawberries	266	29	0	<1
Other berries	9	44	0	0
Grapefruit	70	16	0	0
Lemons	23	9	0	0
Oranges	72	18	0	0
Other citrus fruits	11	64	0	0
Apples	290	42	<1	0
Pears	54	31	0	0
Apricots	42	71	0	2
Cherries	212	45	0	<1
Nectarines	51	22	0	0
Olives	11	100	0	0
Peaches	149	24	2	<1
Plums	61	67	0	0
Bananas	49	84	0	0
Kiwi fruit	90	97	0	0
Pineapples	22	95	0	0
Other tropical fruits	14	93	0	0
Cantaloupe	130	65	0	0
Honeydew	43	67	0	0
Watermelon	69	93	0	0
Other vine fruits	15	15	0	0
Other fruits	8	75	0	0
Fruit juices	15	33	0	0
Fruit toppings & syrups	13	77	8	0
Other fruit products	9	67	0	0
Total	**2168**	**49**	**<1**	**<1**
E. Vegetables				
Corn	89	99	0	0
Garden/green/sweet peas	191	85	3	0
Navy beans	20	90	0	0
String beans	116	47	0	0
Other beans, peas, & corn	50	86	0	0
Cucumbers	100	59	0	7
Eggplant	32	59	3	3
Okra	12	100	0	0
Peppers, sweet	72	74	0	0

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, % Over Tolerance	Samples Violative, % No Tolerance
Peppers, other	17	59	0	0
Pumpkins	23	9	0	4
Squash	81	65	0	0
Tomatoes	178	79	0	0
Other fruits used as vegetables	17	53	0	0
Artichokes	10	90	0	0
Asparagus	74	93	0	0
Bok choy	57	61	0	2
Broccoli	169	80	0	0
Cabbage	178	88	0	0
Cauliflower	117	91	0	0
Celery	99	36	0	0
Chinese cabbage	49	86	0	0
Collards	69	38	0	0
Endive/chicory	56	84	0	4
Kale	78	50	0	1
Lettuce	692	56	<1	<1
Mustard greens	48	29	2	4
Spinach	116	43	0	3
Swiss chard	11	55	0	0
Turnip greens	41	7	0	2
Other leaf/stem vegetables	49	86	0	4
Mushroom/truffle products	29	93	0	0
Carrots	140	74	0	3
Onions, bulb	68	96	1[a]	0
Onions, green	17	76	0	6
Onions, other	12	83	0	0
Parsnips	11	27	0	18
Potatoes	271	66	1	<1
Radishes	27	93	0	0
Red beets	24	83	0	0
Sugar beets	15	73	0	0
Sweet potatoes	91	64	0	0
Turnips	12	50	0	0
Other root/tuber vegetables	16	69	0	0
Beans/peas/corn, dried or paste	120	87	0	0
Vegetables with sauce	29	66	3	3
Other vegetable products	18	61	0	0
Total	**3811**	**68**	**<1[a]**	**<1**

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, % Over Tolerance	Samples Violative, % No Tolerance
F. Other				
Filberts/hazel nuts	15	100	0	0
Peanuts	38	61	0	0
Pecans	25	88	0	0
Other nuts, edible seeds, & related products	7	100	0	0
Vegetable oil seed stock	11	55	0	0
Crude vegetable oil	13	85	0	0
Refined vegetable oil	7	100	0	0
Beverage bases	9	100	0	0
Wine	64	38	0	0
Honey, comb	39	46	0	0
Honey, strained	39	97	0	0
Honey, other	25	96	0	0
Other food sweeteners	8	100	0	0
Baby food, dessert	14	100	0	0
Baby food, fruit juice/drink	124	98	0	0
Baby food, other	17	100	0	0
Other food products	7	100	0	0
Total	**462**	**81**	**0**	**0**
A–F Total	**8281**	**64**	**<1[a]**	**<1**

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

APPENDIX B

Analysis of Import Surveillance Samples by Commodity Group in 1991

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, % Over Tolerance	Samples Violative, % No Tolerance
A. Grains and Grain Products				
Rice	10	90	0	0
Rice products	59	85	0	3
Wheat products	37	51	0	5
Other whole grains	23	74	4	0
Cookies	32	91	0	3
Crackers	12	83	0	0
Other bakery products	45	78	0	2
Cereal and snack foods	35	74	0	6
Macaroni/spaghetti products	57	44	0	0
Noodles/noodle products	50	78	0	0
Other grain products	36	75	0	0
Total	**396**	**72**	**<1**	**2**
B. Milk/Dairy Products				
Total	**216**	**90**	**0**	**0**
C. Fish/Shellfish				
Total	**611**	**77**	**0**	**<1**
D. Fruits				
Blackberries	39	51	0	13
Blueberries	80	81	0	1
Cranberries	17	47	0	0
Grapes	177	40	<1	0
Raspberries	145	44	0	0
Strawberries	155	27	0	10
Other berries	22	45	0	18
Clementines	21	29	0	14
Grapefruit	12	100	0	0
Lemons	53	32	8	0
Limes	11	73	0	0
Oranges	83	57	0	0
Other citrus fruits	7	86	0	0
Apples	113	54	0	0
Pears	236	54	0	0
Other core fruits	8	100	0	0
Apricots	36	64	0	0
Avocados	34	94	0	0
Cherries	51	43	0	0
Nectarines	97	39	1	0
Olives, stuffed	19	84	0	5
Olives, other	77	94	0	1
Peaches	118	35	0	<1
Plums	108	61	0	0
Other pit fruits	9	78	0	0
Bananas	464	70	0	0
Breadfruit	28	96	0	0
Kiwi fruit	19	63	0	0
Mangoes	111	96	0	0
Papaya	55	89	0	0
Passion fruit	46	98	0	0
Pineapples	74	73	0	0
Plantains	35	100	0	0
Other tropical fruits	219	97	<1	1
Bitter melons	22	95	0	0
Cantaloupe	204	44	0	<1
Honeydew	94	61	0	0
Watermelon	95	81	1	1
Other vine fruits	39	82	0	0
Mixed fruits	8	100	0	0
Fruit jams & jellies	30	93	0	0
Fruit juices	76	82	0	0
Fruit toppings	33	85	0	0
Fruits, dried or paste	101	94	0	0
Total	**3481**	**65**	**<1**	**1**
E. Vegetables				
Bean curd/soybeans	15	60	0	7
Corn	28	93	0	0
Garbanzo beans/chick peas	14	79	0	7

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, % Over Tolerance	Samples Violative, % No Tolerance
Garden/green/sweet peas	165	48	4	8
Mung beans	19	95	0	0
Pigeon peas	10	90	0	0
String beans	117	48	0	6
Other beans, peas, & corn	60	63	0	8
Chinese eggplant	40	57	2	5
Cucumbers	131	44	0	3
Eggplant	52	65	0	4
Okra	63	79	0	8
Peppers, hot	431	43	2	3
Peppers, sweet	532	79	<1	<1
Pumpkins	37	92	0	0
Squash	298	59	0	2
Tomatillos	39	54	0	3
Tomatoes	457	64	0	2
Other fruits used as vegetables	68	79	0	6
Asparagus	151	91	<1	0
Bamboo shoots	12	100	0	0
Bok choy	12	42	0	8
Broccoli	66	74	0	2
Broccoli raab	26	69	0	0
Brussels sprouts	14	86	0	0
Cabbage	36	75	0	0
Cauliflower	24	92	0	0
Celery	28	46	0	0
Chicory	61	72	0	2
Chinese broccoli	12	67	0	0
Chinese cabbage	14	79	0	0
Endive/escarole	64	87	0	2
Kale	10	20	0	0
Lettuce	40	60	0	2
Mustard greens	11	55	0	9
Radicchio	140	86	0	5
Spinach	25	64	0	4
Other leaf/stem vegetables	86	64	0	15
Mixed vegetables	6	67	0	0
Mushroom/truffle pieces & products	58	78	9	0
Whole fungi, mushrooms, & truffles	110	78	5	<1
Carrots	65	62	0	2
Cassava	16	94	0	0
Ginger root	11	100	0	0
Leeks	12	83	0	0
Onions, bulb	44	91	0	0
Potatoes	67	72	0	1
Radishes	20	75	0	0
Red beets	12	83	0	0
Rutabagas	12	67	0	0
Scallions	86	51	0	0
Shallots	16	100	0	0
Sweet potatoes	35	97	0	0
Taro	11	91	0	0
Turnips	11	91	0	0
Water chestnuts	20	100	0	0
Other root/tuber vegetables	62	97	0	0
Vegetables, dried or paste	174	80	0	3
Vegetables with sauce	34	97	0	0
Other vegetables/vegetable products	21	86	0	0
Total	**4311**	**68**	**<1**	**3**
F. Other				
Whole spices	25	80	0	4
Other spices, extracts/flavors	12	58	0	8
Cashews	51	63	0	10
Chestnuts	10	100	0	0
Coconuts	11	91	0	0
Peanuts	40	70	0	12
Other nuts	28	93	0	0
Nut products	13	77	0	8
Edible seeds	20	70	0	10
Crude vegetable oil	12	83	0	0
Refined vegetable oil	21	95	0	0
Beverage bases, fruit	24	79	0	0
Coffee & tea	75	93	0	0
Water & ice	38	100	0	0
Wine	471	74	<1	3
Other alcoholic beverages	10	100	0	0
Dressings & condiments	12	67	0	0
Honey	13	100	0	0
Other food products	32	91	0	0
Total	**918**	**79**	**<1**	**3**
A–F Total	**9933**	**69**	**<1**	**2**

APPENDIX C

Pesticide Intakes (μg/kg body wt/day) Found in Total Diet Analyses and Their ADIs/RfDs for 3 Age/Sex Groups in 1991

Pesticide	Age/Sex Group: 6–11 mo.	14–16 yr M[a]	60–65 yr F[a]	FAO/WHO ADI[b]	EPA RfD[b]
Acephate	0.0089	0.0113	0.0165	30	4
Azinphos-methyl	0.0028	0.0033	0.0029	5	–[c]
BHC, alpha	0.0002	0.0004	0.0002	–[c]	–
BHC, gamma (lindane)	0.0004	0.0008	0.0003	8	0.3
Captan	0.0478	0.0209	0.0595	100	130
Carbaryl	0.1801	0.0900	0.0811	10	–
Carbofuran, total	0.0002	0.0001	0.0004	10[d]	5[d]
Chlordane, total	0.0001	0.0003	0.0002	0.5	0.06
Chlorpropham, total	0.1758	0.2205	0.1266	–	200[e]
Chlorpyrifos	0.0082	0.0034	0.0024	10	3
Chlorpyrifos-methyl	0.0104	0.0126	0.0066	1	–
DCPA	0.0002	0.0001	0.0002	–	500
DDT, total	0.0095	0.0056	0.0043	20[d]	0.5[e]
DEF	<0.0001	0.0001	<0.0001	–	–
Demeton	0.0005	0.0003	0.0006	–	0.04
Diazinon	0.0049	0.0022	0.0022	2	–
Dichlorvos	<0.0001	<0.0001	0.0001	4	0.5
Dicloran, total	0.1826	0.0440	0.1175	30[e]	–
Dicofol, total	0.0218	0.0077	0.0235	25	–
Dieldrin	0.0027	0.0021	0.0021	0.1[d]	0.05
Dimethoate	0.0340	0.0022	0.0035	10	0.2
Endosulfan, total	0.0173	0.0158	0.0242	6[d]	0.05[e]
Endrin	<0.0001	<0.0001	<0.0001	0.2	0.3
Ethion	0.0128	0.0034	0.0035	2	0.5
Fenitrothion	<0.0001	<0.0001	<0.0001	5	–
Fenuron[f]	0.0004	0.0002	0.0003	–	–
Fonofos	<0.0001	<0.0001	<0.0001	–	2
Heptachlor, total	0.0003	0.0003	0.0002	0.1[d]	0.5[e]
Hexachlorobenzene	0.0003	0.0004	0.0002	–	0.8
Iprodione, total	0.0026	0.0008	0.0019	300[e]	40[e]
Linuron	0.0021	0.0008	0.0010	–	2
Malathion	0.0779	0.0487	0.0275	20	20
Methamidophos	0.0120	0.0102	0.0190	4	0.05
Methomyl	0.0053	0.0037	0.0068	30	25
Methoxychlor, *p,p'*	0.0006	0.0007	0.0002	100	50
Metobromuron[f]	–[g]	<0.0001	0.0001	–	–
Mevinphos, total	0.0066	0.0026	0.0081	1.5[d]	–
Neburon[f]	<0.0001	<0.0001	<0.0001	–	–
Omethoate	0.0144	0.0013	0.0019	0.3	–
Parathion	0.0097	0.0016	0.0042	5	–
Parathion-methyl	0.0007	0.0001	0.0001	20	–
Pentachlorophenol	0.0016	0.0004	0.0008	–	30
Permethrin, total	0.0251	0.0338	0.0495	50	50
Phosalone	0.0073	<0.0001	<0.0001	6	–
Phosmet	0.0043	0.0009	0.0027	20[d]	20
Pirimiphos-methyl	0.0007	0.0010	0.0006	10	10
Propargite	0.0991	0.0495	0.0491	150	20
Quintozene, total	0.0004	0.0009	0.0003	7[d]	3[e]
Thiabendazole	0.3950	0.2997	0.3062	300	–
Toxaphene	0.0033	0.0059	0.0024	–	–
Vinclozolin	0.0052	0.0018	0.0061	70	25

[a] M = male, F = female.

[b] ADIs and RfDs are usually expressed as mg/kg body wt/day but are expressed here as μg/kg body wt/day for ease of comparison. The ADIs cited here reflect revisions made in 1991. The RfDs cited here reflect May 27, 1992 EPA revisions and are reprinted with the permission of EPA.

[c] ADI or RfD not established.

[d] Includes other (related) chemicals.

[e] Parent chemical only.

[f] On the basis of phenylurea analysis of 14 selected foods in 1 market basket and 20 foods in each of the other 3 market baskets.

[g] No consumption of a food item containing this residue in this age/sex group.

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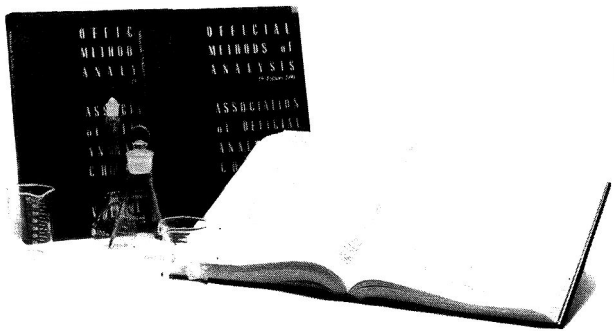

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CHEMICAL CONTAMINANTS MONITORING

Ergot Alkaloids in Grain Foods Sold in Canada

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Six pharmacologically active ergot alkaloids (ergonovine [ergometrine], ergosine, ergotamine, ergocornine, α-ergokryptine, and ergocristine) were determined by liquid chromatography with fluorescence detection in over 400 samples of grain foods. The survey was conducted over a 6-year period for rye flour, wheat flour, and bran/bran cereal, and a 5-year period for rye bread/crispbread; triticale flour was analyzed over a total of 3 years. The predominant alkaloids found were ergocristine and ergotamine. Rye flour was the most contaminated food; the incidence of alkaloid-positive samples was 118/128, annual mean concentrations of total alkaloids in positive samples ranged from 70 to 414 ng/g, and 1 sample contained as high as 3972 ng total alkaloids/g. In wheat flour, total alkaloid concentrations were much lower than in rye flour, with annual means in positive samples (68/93) of 15–68 ng/g. Bran/bran cereal had alkaloid concentrations similar to those in wheat flour, with annual means of 12–69 ng total alkaloids/g positive samples (incidence 29/35). Triticale flour was also an important source of ergot alkaloids; incidence was 24/26, and annual mean total alkaloid concentrations in positive samples were 46–283 ng/g. Ergot alkaloids were found in rye bread (46/100) and other heat-processed rye flour products (6/14). Annual mean total alkaloids in positive rye bread/crispbread samples ranged from 4.8 to 100 ng/g; the latter included a sample with 1248 ng total alkaloids/g.

Contamination of cereal grain with ergot formed by the fungus *Claviceps purpurea* is well-known; rye is the main cereal affected (1). Many countries have strict grading standards for ergot (1). In Canada, there is a maximum limit of 0.05% ergot for the highest grade of rye; for the highest grade of most types of wheat, the limit is 3 kernel-sized pieces/500 g (0.01% in the highest grade of most export grade wheats) (2). Studies with wheat indicate that most of the ergot bodies are removed during cleaning, while as a result of the milling process, the feed streams (bran or shorts depending on the mill used) contained the highest ergot levels (3). However, results from milling ergot-containing rye showed higher concentrations of ergot (determined by its ergometrine content) in the flour fractions relative to bran (4, 5). Thus, low concentrations of the major pharmacologically active ergot alkaloids—ergonovine (ergometrine), ergosine, ergotamine, ergocornine, α-ergokryptine, and ergocristine—might be expected in commercial flour, particularly rye flour. Preliminary Canadian analyses supported this and also indicated that triticale flour could contain high concentrations of ergot alkaloids (up to 375 ng/g) (6–8).

To acquire knowledge of the prevalence and concentrations of ergot alkaloids in the Canadian food supply, a multi-year survey of grain foods for 6 ergot alkaloids was initiated in 1985.

Received December 2, 1991. Accepted February 27, 1992.

Materials and Methods

Samples

Samples (ca 1 kg) were collected from across Canada. Most rye flour and wheat flour samples were obtained at flour mills; triticale flour was collected at mills and health food stores; rye bread was usually obtained at bakeries; bran was obtained from mills and health food stores; and other foods were generally obtained at the retail or wholesale level. Samples were stored in the dark.

Sample Preparation for Breads

Breads were sliced and cubed, and then dried at 22°C for 48–72 h, and the loss of weight was recorded. The dried breads were ground to pass through 2 mm mesh, and analytical results for ergot alkaloids were reported on a fresh weight basis.

Extraction and Cleanup of Samples

Fifty gram samples were extracted and cleaned up as described by Scott and Lawrence (6), with the following minor modifications: shaking time for the extraction step was 10 min;

Table 1. Recoveries of ergot alkaloids from grain foods[a]

Commodity	n		Rec., %						
			Ergonovine	Ergosine	Ergotamine	Ergocornine	α-Ergokryptine	Ergocristine	Total alkaloids
Rye flour	8	Mean	86[b]	75	74	81	80	76	77
		Range	65–104	57–83	53–97	72–94	74–93	61–91	65–91
Rye bread	14	Mean	70[c]	81	71	78	83	80	78
		Range	55–87	56–114	52–100	62–100	72–119	64–107	63–103
Wheat flour	10	Mean	51[d,e]	78	72	78	75	68	71
		Range	24–77	62–93	50–85	70–88	62–90	44–86	58–82
Wheat bran	4	Mean	107[f]	74	72	80	74	77	79
		Range	98–124	69–79	65–80	73–93	70–84	73–87	73–87
Triticale flour	1		79	77	75	80	80	80	79
5-Grain granola	1		79	67	57	74	63	53	67

[a] Spiking levels: 10 ng ergonovine/g sample, 40 ng each other alkaloid/g sample.
[b] 4 of 8 not determined due to interference.
[c] 10 of 14 not determined due to interference.
[d] 1 of 10 not determined due to interference.
[e] 2 of 10 not reported (0% and 3% recovery).
[f] 1 of 4 not determined due to interference.

a 5 min centrifuging (1500 rpm) before filtration of the extract was introduced in 1988; the evaporated extract residue was dissolved in four 10 mL portions of diethyl ether; refrigerated 0.5N HCl was used to reextract the alkaloids; the volume of ammonium hydroxide was 10 mL; and the final residue was dissolved in acetonitrile–methanol (4 + 1, v/v) for liquid chromatographic (LC) analysis (9, 10). Except for rotary evaporation, operations were performed in subdued light.

Liquid Chromatography

Ergot alkaloid standards were obtained from Sandoz A.G., Basel, Switzerland (ergosine, ergocornine maleate, and ergocristine) or from Sigma Chemical Co., St. Louis, MO (ergonovine maleate, ergotamine tartrate, α-ergokryptine, and ergocristine). Standards were dried overnight before use at 70°C in an oven under vacuum. These dried standards retained chromatographic purity and retention time and had an average 18% higher LC response than undried standards; in the few analyses where undried standards were used, appropriate correction factors for the individual alkaloids (ranging from 1.08 to 1.25) were applied. LC standard solutions were prepared from stock solutions and intermediate standard mixture solutions to give 100 ng ergonovine and 400 ng of each of the other alkaloids/mL methanol–acetonitrile (1 + 4, v/v) (10). Standard solutions were stored at −2°C in the dark and prepared weekly (LC and intermediate solutions) or at least monthly (stock solutions). Light was excluded from standards as well as sample extracts during LC analysis.

Ergonovine, ergosine, ergotamine, ergocornine, α-ergokryptine, and ergocristine were determined by LC with fluorescence detection (excitation wavelength, 235 nm). For part of the first year (1985/1986), the analytical column was 10 μm Spheri 10 RP-18 (250 × 4.6 mm, Brownlee Laboratories), used under the following conditions: (*1*) isocratic operation at 1 mL/min with a mixture of 60% 0.0125M KH_2PO_4 buffer (adjusted to pH 6.5 with 0.025M NaOH), and 40% acetonitrile; (*2*) after elution of ergonovine, the flow rate was increased to 2 mL/min; (*3*) after elution of the alkaloids, the LC column was flushed with a mixture of 20% 0.0125M KH_2PO_4 buffer (pH 6.5), and 80% acetonitrile for 10 min; and (*4*) reequilibration at initial conditions for 10 min. Retention times for ergonovine, ergosine, ergotamine, ergocornine, α-ergokryptine, and ergocristine were typically 6.5, 14.5, 16.8, 19.1, 24.1, and 27.3 min, respectively.

During the remainder of 1985/1986 and in subsequent years, the analytical column was Partisil 10 ODS-3 (250 × 4.6 mm, Whatman), used under the following ion-pairing conditions (11): (*1*) isocratic operation at 1 mL/min with a mixture of 60% 0.014M sodium heptanesulfonate in aqueous 1% acetic acid, and 40% acetonitrile–acetic acid (99 + 1, v/v); (*2*) after elution of ergocristine, the column was flushed 10 min with a mixture of 30% 0.014M sodium heptanesulfonate in 1% acetic acid, and 70% acetonitrile–acetic acid (99 + 1, v/v); and (*3*) reequilibration under the initial conditions for 10 min. Typical retention times for ergonovine, ergosine, ergotamine, ergocornine, α-ergokryptine, and ergocristine with this LC system were 4.7, 10.9, 12.4, 13.9, 17.6, and 19.6 min, respectively. In addition to the similar elution patterns, both LC systems gave comparable quantitative responses.

Detection limits for the overall method were 1 ng/g sample (signal to noise, 3:1) for ergonovine and 1 or 2 ng/g sample for the other 5 alkaloids; the higher value was used for the first 2

Table 2. Ergot alkaloids in rye flour

Years analyzed	Incidence (for total alkaloids)	Mean concentration in samples positive for alkaloids (ng/g)[a,b] Ergonovine	Ergosine	Ergotamine	Ergocornine	α-Ergokryptine	Ergocristine	Total alkaloids
1985/1986	12/14	8.4 (38)	5.9 (15)	19 (44)	2.0 (25)	9.5 (36)	36 (163)	80 (297)
1986/1987	20/20	26 (314)	71 (718)	128 (1454)	43 (415)	31 (225)	113 (847)	414 (3972)
1987/1988	27/33	18 (163)	44 (593)	79 (718)	32 (301)	29 (233)	91 (586)	293 (2378)
1988/1989	25/26	14 (74)	14 (99)	40 (255)	20 (229)	26 (271)	75 (586)	188 (1515)
1989/1990	8/8	7.4 (10)	10 (39)	17 (66)	4.7 (9)	6.9 (18)	24 (104)	70 (244)
1990/1991	26/27	19 (42)	20 (61)	52 (211)	13 (59)	30 (154)	90 (514)	223 (987)

	For all samples, total alkaloids only[a] 1985/1986	1986/1987	1987/1988	1988/1989	1989/1990	1990/1991
Mean, ng/g	69	414	239	181	70	215
Median, ng/g	60	99	17	32	43	137
90th percentile, ng/g	216	960	939	583	—	623

[a] Where not detected in a sample, individual alkaloid concentrations were taken as 0 for purpose of calculating means and other parameters.
[b] Maximum concentrations found are given in parentheses.

years of the survey and some of the 1987/1988 analyses. Method recovery was estimated by spiking blank samples with 10 ng ergonovine/g and 40 ng of each of the other alkaloids/g. Survey data were not corrected for method recoveries.

Results and Discussion

The method of analysis used for this survey generally worked well. The occasional emulsion formed during extraction of the ether phase with 0.5N hydrochloric acid could usually be broken by adding 3 mL methanol and using a stirring rod; refrigerated hydrochloric acid also helped. The LC mobile phase containing phosphate buffer, used in 1985/1986, caused serious column degradation problems and was replaced by an ion-pairing solvent system (11), which also improved peak resolution. Method recoveries were acceptable in most cases. The set of recoveries performed in 1987/1988 is presented in Table 1. Ergonovine was sometimes difficult if not impossible to measure due to complete masking of the peak by co-extractives, particularly with some samples of rye bread. Also, recoveries of ergonovine from a few samples of wheat flour were

Table 3. Ergot alkaloids in wheat flour

Years analyzed	Incidence (for total alkaloids)	Mean concentration in samples positive for alkaloids (ng/g)[a,b] Ergonovine	Ergosine	Ergotamine	Ergocornine	α-Ergokryptine	Ergocristine	Total alkaloids
1985/1986	12/19	2.5 (5.7)	2.1 (8.3)	2.9 (20)	0.5 (6.0)	1.5 (9.3)	5.8 (31)	15 (75)
1986/1987	18/19	4.1 (16)	4.6 (44)	8.7 (64)	3.5 (24)	3.8 (19)	14 (63)	39 (215)
1987/1988	15/23	1.8 (4.8)	3.1 (24)	10 (56)	2.9 (13)	4.6 (27)	17 (64)	39 (163)
1988/1989	9/14	4.1 (10)	1.4 (5.9)	6.6 (18)	2.9 (13)	2.4 (12)	8.8 (32)	26 (85)
1989/1990	4/5	4.9 (10)	11 (42)	20 (63)	3.6 (12)	6.8 (24)	22 (73)	68 (244)
1990/1991	10/13	1.1 (2.0)	1.0 (2.4)	3.1 (5.2)	0.7 (2.6)	1.2 (3.9)	7.6 (16)	15 (25)

	For all samples, total alkaloids only[a] 1985/1986	1986/1987	1987/1988	1988/1989	1989/1990	1990/1991
Mean, ng/g	9.6	37	26	17	54	11
Median, ng/g	1.4	4.3	3.1	4.8	17	14
90th percentile, ng/g	33	129	125	75	—	24

[a] Where not detected in a sample, individual alkaloid concentrations were taken as 0 for purpose of calculating means and other parameters.
[b] Maximum concentrations found are given in parentheses.

Table 4. Ergot alkaloids in bran/bran cereal

Years analyzed	Incidence (for total alkaloids)	Mean concentration in samples positive for alkaloids (ng/g)[a,b] Ergonovine	Ergosine	Ergotamine	Ergocornine	α-Ergokryptine	Ergocristine	Total alkaloids
1985/1986	4/5	7.4 (18)	3.0 (12)	1.6 (6.5)	0.9 (3.6)	ND[c]	1.9 (5.4)	15 (42)
1986/1987	7/9	2.5 (3.5)	1.9 (5.9)	3.9 (9.2)	0.6 (4.5)	0.7 (5.0)	7.1 (14)	17 (40)
1987/1988	8/9	4.1 (12)	2.7 (11)	7.7 (27)	4.6 (13)	5.8 (16)	19 (47)	44 (119)
1988/1989	3/4	7.4 (8.8)	0.8 (2.3)	7.7 (11)	3.8 (4.8)	2.0 (3.7)	16 (21)	38 (49)
1989/1990	1/2	5.1	ND[c]	2.4	1.4	ND[c]	2.6	12
1990/1991	6/6	4.3 (7.7)	4.1 (11)	11 (24)	8.4 (38)	12 (39)	29 (51)	69 (168)

For all samples, total alkaloids only[a]

	1985/1986	1986/1987	1987/1988	1988/1989	1989/1990	1990/1991
Mean, ng/g	12	13	39	28	5.8	69
Median, ng/g	7.3	14	20	32	—	45

[a] Where not detected in a sample, individual alkaloid concentrations were taken as 0 for purpose of calculating means and medians.
[b] Maximum concentrations found are given in parentheses.
[c] Not detected.

very low, although recoveries of the other simultaneously added ergot alkaloids were good.

Results of the survey, on an annual basis, are presented in Tables 2–6. The means and maxima in alkaloid-positive samples are shown for each alkaloid and total alkaloids, as well as incidences for total alkaloids. In addition, for total alkaloids only, means, medians, and, where appropriate, 90th percentiles have been calculated for all samples. A cumulative summary is provided in Table 7. The observed differences in annual means for all samples of a particular commodity (Tables 2–6) illustrate the importance of performing a multi-year survey to obtain a better estimate of ergot alkaloid concentrations in grain foods (Table 7). The most commonly occurring alkaloids found were ergotamine and ergocristine, in agreement with published studies on alkaloid composition of Canadian rye, wheat, and triticale ergot sclerotia (12–15).

Confidence in identification of the alkaloids was derived from (*1*) co-occurrence of several or all of the 6 alkaloids in the majority of alkaloid-positive samples, (*2*) to a degree, the relative concentrations of the individual alkaloids, and (*3*) the generally uncluttered chromatograms (apart from the ergonovine interference problem mentioned above). Consideration was given to regarding as negative those survey samples containing low levels of ergonovine with no other detectable alkaloids. These comprised 2 samples of rye flour (mean, 4.1 ng ergonovine/g), 18 samples of wheat flour (mean, 3.0 ng ergonovine/g), 2 samples of triticale flour (mean, 1.3 ng ergonovine/g), 3 samples of rye bread (mean, 3.5 ng ergonovine/g),

Table 5. Ergot alkaloids in triticale flour

Years analyzed	Incidence (for total alkaloids)	Mean concentration in samples positive for alkaloids (ng/g)[a,b] Ergonovine	Ergosine	Ergotamine	Ergocornine	α-Ergokryptine	Ergocristine	Total alkaloids
1985/1986	10/12	5.6 (13)	2.2 (10)	9.9 (34)	5.3 (20)	5.3 (26)	25 (81)	54 (183)
1986/1987	10/10	4.3 (13)	5.2 (21)	10 (38)	4.1 (27)	3.9 (27)	19 (84)	46 (210)
1990/1991	4/4	25 (41)	17 (30)	49 (99)	24 (53)	43 (82)	125 (241)	283 (545)

For all samples, total alkaloids only[a]

	1985/1986	1986/1987	1990/1991
Mean, ng/g	45	46	283
Median, ng/g	19	35	231
90th percentile, ng/g	173	195	—

[a] Where not detected in a sample, individual alkaloid concentrations were taken as 0 for purpose of calculating means and other parameters.
[b] Maximum concentrations found are given in parentheses.

Table 6. Ergot alkaloids in rye bread/crackers/crispbread

Years analyzed	Incidence (for total alkaloids)[c]	Mean concentration in samples positive for alkaloids (ng/g)[a,b]						
		Ergonovine	Ergosine	Ergotamine	Ergocornine	α-Ergokryptine	Ergocristine	Total alkaloids
1986/1987	16/20	5.2 (67)	24 (318)	43 (545)	11 (136)	3.2 (29)	14 (153)	100 (1248)
1987/1988	11/39 (10/35)	2.3 (10)[d]	4.1 (26)	5.8 (26)	3.6 (12)	3.5 (14)	11 (46)	30 (95)
1988/1989	15/30 (14/26)	4.1 (26)[e]	2.8 (15)	4.8 (24)	1.7 (7.6)	3.2 (12)	8.1 (24)	22 (82)
1989/1990	4/6	<1	1.6 (3.5)	1.6 (3.5)	0.3 (1.0)	0.5 (1.0)	1.0 (1.6)	4.8 (11)
1990/1991	6/19 (2/13)	1.8 (7.0)	0.5 (3.1)	2.7 (5.2)	1.3 (4.9)	0.7 (3.2)	1.5 (2.9)	8.5 (20)

	For all samples, total alkaloids only[a]				
	1986/1987	1987/1988	1988/1989	1989/1990	1990/1991
Mean, ng/g	80	8.5	11	3.2	2.7
Median, ng/g	6.3	0	1.1	2.3	0
90th percentile, ng/g	113	55	45	—	12

[a] Where not detected in a sample, individual alkaloid concentrations were taken as 0 for purpose of calculating means and other parameters.
[b] Maximum concentrations found are given in parentheses.
[c] Incidences for rye bread only are given in parentheses.
[d] Based on 9 samples only; not measurable in 2 alkaloid-positive samples (rye bread).
[e] Based on 7 samples only; not measurable in 8 alkaloid-positive samples (rye bread). Therefore, the mean for total alkaloids is an underestimation.

and 3 bran samples (mean, 3.7 ng ergonovine/g). However, ergot sclerotia whose major alkaloid was ergonovine have been reported (12), so these contributors to positive samples are included in Tables 2–7.

Rye flour contained the highest concentrations of ergot alkaloids of all the foods surveyed (Tables 2 and 7). Mean total alkaloid concentrations in positive rye flour samples exceeded 100 ng/g in 4 of the 6 years of analysis (Table 2). The 2 most contaminated samples were rye flours from Quebec that, respectively, contained 314 ng ergonovine, 718 ng ergosine, 1454 ng ergotamine, 415 ng ergocornine, 225 ng α-ergokryptine, and 847 ng ergocristine/g flour (3972 ng total alkaloids/g); and 92 ng ergonovine, 593 ng ergosine, 718 ng ergotamine, 301 ng ergocornine, 185 ng α-ergokryptine, and 489 ng ergocristine/g flour (2378 ng total alkaloids/g).

Wheat flour had consistent but lower levels of alkaloid contamination. For all 6 years of analysis, the annual mean total alkaloid concentrations in positive wheat flour samples did not exceed 100 ng/g (Table 3). Over the 6-year period, the overall mean concentrations of total alkaloids in positive and all samples, as well as the overall median concentration in all samples, were an order of magnitude lower than the corresponding values for rye flour (Table 7). The 2 wheat flour samples with the highest concentrations of alkaloids (from Quebec), respectively, contained 10 ng ergonovine, 42 ng ergosine, 63 ng ergotamine, 12 ng ergocornine, 24 ng α-ergokryptine, and 73 ng ergocristine/g flour (224 ng total alkaloids/g); and 5.4 ng ergonovine, 44 ng ergosine, 64 ng ergotamine, 24 ng ergocornine, 15 ng α-ergokryptine, and 63 ng ergocristine/g flour (215 ng total alkaloids/g).

Thirty-one samples of bran and 4 bran cereal samples were analyzed over the 6-year period. Low concentrations of ergot alkaloids, similar to those in wheat flour, were detected. The highest mean annual concentration of total alkaloids was 69 ng/g sample (Table 4); this figure included the most contaminated bran sample, which contained 5.2 ng ergonovine, 11 ng ergosine, 24 ng ergotamine, 38 ng ergocornine, 39 ng α-ergokryptine, and 51 ng ergocristine/g sample (168 ng total alkaloids/g).

Triticale flour was also an important source of ergot alkaloids, although in only 1 of the 3 years studied was the mean concentration of total alkaloids greater than 100 ng/g (Table 5). The most contaminated triticale flour sample had a total alkaloid concentration of 545 ng/g (see Table 5 for the concentrations of individual alkaloids in this sample).

Of particular interest to consumers are concentrations of ergot alkaloids found in bread. We have detected low levels in rye bread and other heated rye flour products, and in 1986/1987, the mean level of total alkaloids in positive samples reached 100 ng/g sample (Table 6). This figure was biased, however, by a rye bread sample (again from Quebec) that contained a total alkaloid concentration of 1248 ng/g (concentrations of individual alkaloids in this sample are the maxima shown in Table 6). It is known that the pharmacologically active alkaloids are not heat-stable, but are converted initially to inactive isomers (16). Several published baking studies have indicated alkaloid losses ranging from 33 to 42% for ergonovine added to wheat flour and baked into muffins (17); 45–60% for losses of ergonovine (ergometrine) in ergot during various wheat/rye bread baking operations (18); 47–69% losses of the 6 commonly occurring pharmacologically active alkaloids in added ergot during the process of baking rye bread (19); 63–84% losses of these alkaloids in another study of the same process (20); and up to 74, 85, and 100% losses during pancake

Table 7. Ergot alkaloids in grain foods: 6-year summary

Commodity	Years analyzed	Incidence (for total alkaloids)	Mean concentration in samples positive for alkaloids (ng/g)[a,b] Ergonovine	Ergosine	Ergotamine	Ergocornine	α-Ergokryptine	Ergocristine	Total alkaloids
Rye flour	1985–1991	118/128	17 (314)	31 (718)	63 (1454)	22 (415)	25 (271)	81 (847)	239 (3972)
Wheat flour	1985–1991	68/93	2.9 (16)	3.3 (44)	7.5 (64)	2.3 (24)	3.2 (27)	12 (73)	31 (224)
Bran/bran cereal	1985–1991	29/35	4.6 (18)	2.5 (12)	6.5 (27)	3.7 (38)	4.4 (39)	15 (51)	37 (168)
Triticale flour	1985–1987 1990–1991	24/26	8.4 (41)	5.9 (30)	17 (99)	8.0 (53)	11 (82)	39 (241)	89 (545)
Rye bread/ crackers/ crispbread	1986–1991	52/114	3.4 (67)[c]	9.4 (318)	16 (545)	4.8 (136)	2.8 (29)	9.2 (153)	45 (1248)

For all samples, total alkaloids only[a]	Rye flour	Wheat flour	Bran/bran cereal	Triticale flour	Rye bread/crackers/crispbread
Mean, ng/g	220	23	30	82	21
Median, ng/g	67	4.3	15	33	0
90th percentile, (ng/g)	601	81	110	246	36

[a] Where not detected, individual alkaloid concentrations were taken as 0 for calculating means and other parameters. Detection limits were 1 ng/g for ergonovine and 1–2 ng/g for the other alkaloids.
[b] Maximum concentrations are given in parentheses.
[c] Based on 42 samples; not measurable in 10 alkaloid-positive samples (rye bread).

making from triticale flour, baking rye bread, and baking whole wheat bread, respectively (7). Thus, our survey results for rye bread are not unexpected.

Certain other foods were also analyzed, but low sample numbers do not warrant tabulation. Rye meal and rye flakes were highly contaminated, with an incidence of 8/8 and total alkaloid concentrations of 103–1043 ng/g sample. Three of 3 multi-grain cereals averaged 70 ng total alkaloids/g; these included a granola (muesli) sample with 17 ng total alkaloids/g. In view of a German report of poisoning from consumption of ergot-contaminated muesli (21), this type of food product warrants consideration in future ergot alkaloid surveys.

Few surveys of grain foods for ergot alkaloids have been reported previously. Lu et al. (22), using LC with UV detection, determined that 5 of 66 samples of flour in Taipei, Taiwan, contained ergotamine (57–193 ng/g). Baumann et al. (20), using LC with fluorescence detection, found mean concentrations of total ergonovine (ergometrine), ergosine, ergotamine, ergocornine, α-ergokryptine, and ergocristine to be 93 ng/g in rye flour (6 samples) in Switzerland, 60 ng/g in coarsely ground wheat flour (8 samples), and 11 ng/g in rye bread (5 samples). They also determined the inactive isomers and estimated an intake of both kinds of alkaloids by the Swiss population to be 5.1 μg/person/day (0.08 μg/kg body weight/day). The preliminary surveys of Scott and Lawrence (6, 8) found mean total alkaloids of 63 ng/g in rye flour (6/6 samples), 262 ng/g in triticale flour (2/2 samples), and <20 ng/g in wheat flour (16/16 samples); the alkaloids were also determined by LC with fluorescence detection.

The present survey provides data that should assist in estimating human intake of ergot alkaloids in Canada, particularly from rye products. Such an exposure estimate taken in conjunction with estimates of tolerable daily intake would then indicate whether there is any human health risk from consumption of ergot alkaloids and whether further control measures are required.

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DAIRY PRODUCTS

Assessment of Fourier Transform Infrared Analysis of Milk

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A Nicolet 510 Fourier transform infrared (FTIR) spectrometer was modified to perform IR milk analyses by incorporating a temperature-controlled 37 μm CaF_2 flow cell and a homogenizer into the analytical system. The unit was evaluated for its ability to predict the chemical values of calibration milks, and its performance was compared with that of a commercial filter-based IR milk analyzer (Multispec MK1). Conventional dual-wavelength multiple regression methods and whole-spectrum multivariate analysis techniques (classical least squares and partial least squares) were also compared for their predictive capabilities. We found that the prototype FTIR unit met AOAC standards for milk analyses and performed as well as or better than the filter instrument. The macro-programming capability of the FTIR software, which enables the user to combine strings of FTIR commands and parameters into a single command, allowed automation of data processing. The whole-spectrum multivariate analysis methods were able to provide total solids data directly in addition to fat, protein, and lactose. On the basis of this evaluation, FTIR appears to be a viable alternative means for performing milk analyses.

One of the most noteworthy applications of quantitative mid infrared (IR) spectroscopy has been for milk analysis (1, 2), which has evolved into a standard AOAC method (3) and is used extensively as a basis for milk payment (4), herd milk analysis (5), and routine quality control in the dairy industry (6). Originally developed from modified dispersive instruments (7), the technology quickly moved to the use of interference filters to increase energy and facilitate sample throughput (6, 8). IR milk analyzers generally have a single 37–40 μm CaF_2 flow cell; quantitation is achieved by using 2 wavelengths (reference and sample) per component to be measured. The analyzers are capable of analyzing approximately 1 milk sample every 20 s in automated versions of the instrumentation (8). Such instruments have replaced tedious and time-consuming chemical methods such as the Kjeldahl for protein, Mojonnier for fat, and polarimetry for lactose. This analytical technology has not evolved into more general food applications, although attempts have been made to expand IR applications to meat (9–11), fish (12), carbohydrates (13), lipids (14), moisture (15), and ammonia analyses (16) by using conventional IR milk analyzers. No significant innovations or advances have been made instrumentally or technologically in the field of milk analysis since the mid-1980s (17).

Although conventional dispersive mid IR spectroscopy has long been used as a primary tool for the identification of chemical compounds and has evolved into many forms and specialized applications (18), the utility of IR spectroscopy in quantitative analysis was markedly enhanced by the development of Fourier transform infrared (FTIR) spectroscopy (19) in the early 1970s. Aside from extensive computing and data manipulation capabilities, FTIR also has a number of other advantages, i.e., improved signal-to-noise ratio, multiplexing, a significant reduction in scan times, higher energy throughput, and superior wavelength accuracy (20).

The strength of mid IR analysis lies in the wealth of detail to be found in this portion of the spectrum, making the identification of individual components in a complex mixture relatively simple. In the chemical industry, FTIR in combination with appropriate data-processing software has been cited (21) as a major step forward, providing a new dimension of speed, precision, and accuracy previously not available in quality control laboratories. This paper presents an exploratory investigation of the application of FTIR spectroscopy to the analysis of milk and an assessment of its performance in comparison with that of conventional filter-based instrumentation.

Experimental

Instrumentation

A Nicolet 510 FTIR spectrometer (Nicolet Instrument Corp., Madison, WI 53744) run with Nicolet DXFTIR software was used for this study. A conventional 37 μm CaF_2 flow cell was housed in the sample compartment, fed by a standalone high-pressure homogenizer of the type normally used with a Multispec MK1 IR milk analyzer. Both the homogenizer and cell were heated to 40 ± 1°C with heating tape controlled by 2 Omega CN 9000 (Omega Engineering Ltd, Stamford, CT) feedback temperature controllers. Programs were written to au-

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tomate data collection by using the macro-programming facilities available through the DXFTIR software. Subsequently, scan parameters (mirror velocity, gain, resolution, and number of scans per sample) were optimized, wavelengths for calibration were selected, and calculation protocols were established. All recorded interferograms were automatically converted to emittance spectra by fast Fourier transformation and then stored to disk for subsequent analyses and manipulations.

In all cases, before the spectra of a set of milk standards or samples were recorded, an emittance spectrum of the empty cell was recorded, followed by an emittance spectrum of water, and these were then ratioed to produce an absorbance spectrum of water. The emittance spectra of milk standards or samples were ratioed against the emittance spectrum of the empty cell and against that of water to provide 2 sets of absorbance spectra, one set having water in the spectrum and the other containing only the absorptions due to milk components as well as some residual water absorptions. To determine whether spectra with water present or ratioed out should be used, exploratory quantitative analyses were carried out. Once satisfactory algorithms and methodology were developed, they were incorporated into macros to present quantitative data directly.

Calibration

Calibrations were developed by using commercial calibration powders (Cal-Eze, Glengarry Biotech Inc., Cornwall, ON, Canada), which were reconstituted to produce standard calibrants with known and reproducible chemical values (22, 23). All calibrations and sample handling techniques were carried out according to AOAC procedures (3). Calibrations were developed by using the spectra of 12 reconstituted calibration milks recorded in duplicate.

Data Analysis

Three multivariate methods of analysis were used to develop calibrations from the IR data for the calibration standards: (*1*) a multiple linear regression (MLR) method based on the conventional dual-wavelength ratio method normally used in filter-based IR milk analyzers; (*2*) the classical least-squares (CLS) method, based on the matrix formulation of Beer's law [K-matrix method (24)], whereby the absorbance at each wavelength in the spectrum is related to the concentrations of the components in the sample through a matrix of coefficients determined in the calibration step; and (*3*) the partial least-squares (PLS) method (25), a form of factor analysis. A detailed description of the mathematical basis of the PLS method is available elsewhere (26) and is beyond the scope of this paper. MLR was performed using the Statgraphics statistical software package (STSC, Inc., Rockville, MD) and was run without a constant to force the regression through 0, because this procedure produces more consistent linear regression equations. The CLS and PLS methods were implemented by software packages available from Nicolet Analytical Instruments. In the Nicolet implementation of the PLS algorithm, the optimum rank of the PLS model for each component is determined by using a cross-validation procedure in which the mean prediction error for a set of validation samples is measured as a function of rank (25).

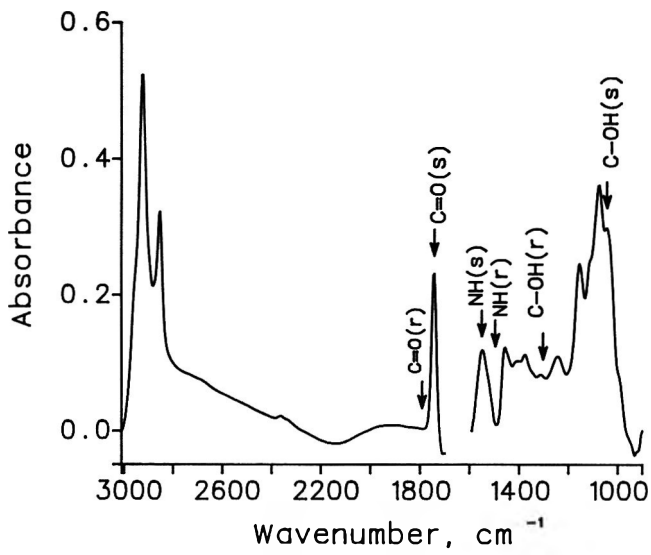

Figure 1. Typical spectrum of milk obtained by FTIR spectroscopy, indicating the sample (s) and reference (r) wavelengths used for quantitation of fat (C=O), protein (NH), and lactose (C–OH) by MLR. Scan parameters: mirror velocity = 30, gain = 1, resolution = 16 cm^{-1}, number of scans = 30. The water absorptions have been eliminated by ratioing the emittance spectrum of the sample against the emittance spectrum of water. The region between 1720 and 1600 cm^{-1} has been blanked because of the intense water absorption in this region, which cannot be properly ratioed out.

For the MLR method, the following sample/reference wavelength pairs were used: fat A or ester linkage (1744.6/1792.1 cm^{-1}), protein or amide II (1548.6/1497.0 cm^{-1}), and lactose or C–OH (1043.1/1302.1 cm^{-1}). CLS and PLS are whole-spectrum methods, although in practice the analysis is restricted to regions of the spectrum that exhibit variations with changes in the concentrations of the components of interest. Both the CLS and PLS programs take as input the concentrations of the reference standards and the spectral regions selected for the analysis on the basis of their information content with respect to the concentrations of the components. Both programs retrieve the spectra of the reference standards previously recorded on disk to develop the calibration automatically by using the reference concentration values provided for the calibration samples. The programs also store the resulting calibration matrix. In PLS, a portion of the calibration samples are used as validation samples. The designated validation samples are not used in the construction of the calibration model but are used to assess the predictive ability of the calibration and to determine the optimum rank of the PLS model for each component. The calibration models obtained from both CLS and PLS can be called from a macro program to convert spectral data for unknown samples to component concentrations directly.

Table 1. Representative multiple linear regression coefficients (C) and normalized coefficients (NC), coefficients of multiple determination (R^2), and standard errors (SE) for the calibration equations obtained for the Multispec and FTIR procedures[a]

	Multispec		FTIR	
Parameter	C	NC	C	NC
		Fat		
F_i	0.9894	1.0000	18.14	1.0000
P_i	0.0185	–0.0187	0.05	0.0027
L_i	–0.0004	–0.0004	0.23	0.0126
R^2	0.9998	—	0.9999	—
SE	0.044	—	0.040	—
		Protein		
F_i	0.0272	0.0273	1.28	0.0637
P_i	0.9950	1.0000	20.08	1.0000
L_i	0.0765	0.0768	0.45	0.0224
R^2	0.9999	—	0.9999	—
SE	0.040	—	0.042	—
		Lactose		
F_i	–0.0175	–0.0188	1.14	0.0655
P_i	0.0106	0.0114	2.07	0.1189
L_i	0.9267	1.0000	17.40	1.0000
R^2	0.9999	—	0.9998	—
SE	0.045	—	0.078	—

[a] n = 12. *F*, *P*, and *L* represent fat, protein, and lactose, respectively.

The predicted values for the reference standards obtained from each of the 3 calibration methods were compared with the chemical reference values of the calibration standards according to AOAC methods (27) in terms of mean differences for accuracy (MD_a) and standard deviations of the differences for accuracy (SDD_a).

Results and Discussion

The basic instrumental parameters chosen for this work were as follows: mirror velocity = 30, gain = 1, resolution = 16 cm^{-1}, and number of scans per sample = 12. Initial work was carried out using 50 scans/sample, because noise is inversely proportional to the square root of the number of scans taken; however, we found that a satisfactory signal-to-noise ratio was obtained with 12 scans.

In preliminary work, we established that the accuracy of quantitative analysis based on the MLR method, as described above, was improved when water was removed from the calibration spectra by ratioing against the spectrum of water. In the case of the CLS and PLS methods, water does not have to be removed from the spectra.

The FTIR spectrum of a calibration milk ratioed against the spectrum of water is shown in Figure 1; the sample/reference wavelength pairs used in the MLR approach are indicated on the figure with arrows. The peak heights measured at these wavelengths were ratioed and related to concentration by MLR. For purposes of comparison, the reference standards were also analyzed by a Multispec MK1 IR milk analyzer. Table 1 presents representative coefficients and regression statistics obtained from the Multispec and FTIR data for equations of the form:

$$F_{ce} = aF_i + bP_i + cL_i$$
$$P_{ce} = dF_i + eP_i + fL_i$$
$$L_{ce} = gF_i + hP_i + iL_i$$

where *F*, *P*, and *L* represent fat, protein, and lactose, respectively, and the subscripts ce and i represent the chemical estimates predicted and the instrumental values recorded, respectively. Although the equations are not directly comparable, because the Multispec uses a slope setting to bring the absorbance values into the true component percentage range, they can be compared if the primary coefficient in each equation is normalized to 1.0 and the other coefficients are adjusted accordingly. The normalized coefficients obtained in this way are also given in Table 1. In this format, it can be seen that the basic forms of the equations from the 2 different instruments are very similar, with the major contributing coefficient in each case corresponding to the component being determined and other components making a small contribution related to displacement and cross-absorbance effects.

Table 2 presents typical predictions obtained by using the regular linear regression equations along with the MD_a and SDD_a for the 12 calibration milks. The MD_a and SDD_a are generally well within the AOAC specifications for milk analysis (<0.06%), although lactose is just outside the limit in this run. Overall, the fact that the results from the FTIR analysis and from the Multispec analysis are comparable indicates that the conventional MLR-based dual-wavelength method works well with FTIR.

Attempts were also made to improve the results of the FTIR analysis through the use of peak areas. In general, this approach resulted in poorer predictions (i.e., higher MD_a and SDD_a). Results similar to those obtained with the dual-wavelength method could be obtained from combinations of dual-wavelength and peak area data by stepwise multiple regression. Stepwise regression often resulted in widely differing equations; the variables determined to be significant changed between calibration trials. Although each equation might well predict similar values, it is difficult to establish whether changes in the equations are a result of a fundamental change in the data or are due to the regression procedure itself. Hence, the consistency from run to run of the equations obtained with the 3 sample/reference wavelength pairs makes it a more suitable form of analysis, and little appears to be gained by providing additional information through the use of peak areas.

The other 2 multivariate methods of analysis investigated were CLS and PLS. Both of these are full-spectrum methods and, thus, can provide significant improvements in precision relative to methods that use only a limited number of wavelengths (28). Because CLS is based on Beer's law and, thus, on

Table 2. Reference chemical values for the 12 calibration milks (CHEM) and typical instrumental predictions obtained from the Multispec MK1 (MULT) and Nicolet 510 (FTIR) and their relative MD_a and SDD_a

	Fat, %			Protein, %			Lactose, %		
Sample	CHEM	MULT	FTIR	CHEM	MULT	FTIR	CHEM	MULT	FTIR
1	0.10	0.08	0.04	3.43	3.50	3.46	5.53	5.56	5.47
2	3.15	3.12	3.14	3.09	3.12	3.13	4.91	4.88	4.90
3	4.51	4.42	4.47	2.62	2.56	2.57	4.01	3.99	3.92
4	2.41	2.40	2.44	3.35	3.35	3.35	5.23	5.20	5.18
5	2.59	2.58	2.59	3.84	3.83	3.85	4.53	4.57	4.54
6	2.54	2.53	2.57	3.54	3.49	3.50	5.12	5.02	5.01
7	3.68	3.65	3.70	3.53	3.54	3.57	3.84	3.78	3.76
8	5.39	5.47	5.42	2.94	2.95	2.93	4.65	4.66	4.67
9	5.86	5.86	5.81	3.07	3.10	3.10	4.92	4.95	4.98
10	1.11	1.14	1.14	3.15	3.09	3.16	4.85	4.83	4.88
11	2.12	2.12	2.15	3.49	3.44	3.44	5.36	5.40	5.45
12	3.03	3.06	2.99	4.59	4.59	4.53	5.21	5.25	5.30
MD_a		0.001	0.000		−0.005	0.000		0.001	−0.006
SDD_a		0.039	0.036		0.040	0.037		0.045	0.068

the assumption that absorbance is linearly related to concentration, all species that absorb in the spectral region of interest must be identified and included in the calibration. PLS does not suffer from this disadvantage, because it treats concentration rather than spectral intensity as the independent variable. Thus, PLS is able to compensate for unidentified sources of spectral interference, although they must be present in both the calibration standards and the samples to be analyzed. In addition, both CLS and PLS are able to account for consistent variations in the spectra, such as baseline shifts.

The PLS calibration method gave predicted values for the reference standards with MD_a and SDD_a similar to those obtained from the conventional MLR procedure (Table 3). CLS performed comparably for fat and lactose, but did not do as well for protein (Table 3).

Both PLS and CLS potentially provide the capability to determine the total solids (TS) content directly. Because the dry calibration powders are reconstituted on a specified weight basis, the TS content is known. Because both CLS and PLS work with water present in the spectra, provided regions of intense water absorption (>2 absorbance units) are blanked, water can be included as an additional component (% water = 100% − %TS) to be determined in these analyses, directly yielding predictions of the TS content (100% − % water). Such data cannot be obtained from the Multispec except by summation or through the use of a water filter (29), which has not been commercially developed to date for milk applications. Summation ignores any variations in the contributions of minerals or any other unmeasured components to the TS content and compounds any errors associated with the determination of each component (fat, protein, and lactose).

Table 3. MD_a and SDD_a for the calibration milks using CLS and PLS

	CLS		PLS	
Component	MD_a	SDD_a	MD_a	SDD_a
Fat	0.000	0.023	0.006	0.021
Protein	−0.021	0.111	0.005	0.033
Lactose	0.000	0.050	0.006	0.046

Table 4 presents typical predictions obtained from the Multispec summation procedure and from the PLS and CLS analyses, relative to the reference TS value. It can be seen that the results from the summation procedure are well within the AOAC specifications for TS content (MD_a, <0.09%; SDD_a, <0.12%), but that PLS performs better in terms of SDD_a. This would be expected because PLS directly determines the water content, and, hence, the TS content, whereas the summation procedure treats the mineral component, which is not measurable, as a constant. Although the CLS method also allows for the direct determination of the water content, the predicted TS values have an unacceptably high SDD_a (Table 4). This result, combined with the relatively high SDD_a value obtained with CLS for protein (Table 3), indicates that the spectral overlap between the intense water absorption centered at approximately 1640 cm^{-1} and the protein amide I band and, to a lesser extent, the amide II band cause difficulties in the CLS analysis. The superior performance of the more powerful PLS method exemplifies its greater suitability for multicomponent analysis of complex samples.

Thus, both the MLR and PLS methods have been applied successfully to the calibration of an FTIR spectrometer for milk analysis, with PLS offering superior performance in terms of TS predictions. Both the MLR equations and the PLS calibration model obtained with the calibration standards can be incorporated into macro programs, providing immediate predic-

Table 4. Total solids (TS) reference values, total solids determined by summation (fat, protein, and lactose), and predictions for total solids from PLS and CLS[a]

Sample	Reference TS	Summation TS	PLS TS	CLS TS
1	10.0	9.90	10.01	9.47
2	12.0	12.02	12.04	11.91
3	12.0	11.94	11.96	11.91
4	12.0	12.05	12.01	12.27
5	12.0	12.04	11.98	12.09
6	12.0	12.10	12.01	12.47
7	12.0	11.89	12.05	11.67
8	14.0	13.99	14.02	14.76
9	15.0	15.01	15.01	14.33
10	10.0	9.97	9.99	10.34
11	12.0	12.01	12.01	11.71
12	14.0	14.03	13.99	14.09
MD_a		−0.004	−0.006	−0.002
SDD_a		0.062	0.025	0.420

[a] Determined by subtracting % water from 100.

tions as unknown samples are being analyzed by the instrument. For long-term utility of a calibration, allowances have to be made for subtle changes in instrumental variables and cell characteristics (e.g., protein buildup) over time. Such changes are manifested by shifts in the coefficients of the MLR equations and can be readily adjusted for. Because of the complexity of the PLS calibration model, frequent recalibration is impractical for PLS; also, recalibration does not yield readily interpretable information about the nature of any changes that may have occurred over time. To avoid frequent recalibration, one way of adjusting for minor calibration shifts over time is to rerun the standards and perform a simple linear regression of the predicted data obtained from the PLS calibration model against the chemical values. This approach was incorporated directly into our utility program, permitting interactive adjustment of the slope and intercept of the correction equation as required.

Although the instrument used in the present study is a prototype and not equipped for automated sample handling, it would not require much modification to produce an instrument with operating characteristics comparable with those of conventional filter-based instruments. On the basis of our manual operating procedures, we estimate that, with automated sample handling and appropriate programming, an instrument capable of handling up to 300 four-component analyses (fat, protein, lactose, and TS) per hour could readily be produced with appropriate engineering. With optimization, up to 600 samples/h could be handled; however, this in itself might not be practical for many centralized laboratories if the component analyses are tied into somatic cell counting. For situations in which this is not a problem or in which 1 instrument would feed 2 somatic cell counters, 1 FTIR unit could replace 2 conventional filter-based analyzers and provide direct TS analyses without any loss of speed, which would not be the case if a filter instrument was to be equipped with a water filter.

Conclusion

FTIR spectroscopy is clearly applicable to milk analysis, because it provides results comparable with those obtained with conventional IR milk analyzers. Among the 3 forms of multivariate analysis used for the calculation of component concentrations, the MLR and PLS approaches performed comparably, whereas the CLS method showed a poorer predictive capability. The dual-wavelength MLR approach is the simplest method of calculating component concentrations; it provides a set of conventional equations rather than the complex calibration model associated with the PLS method. The latter technique has the advantage of providing direct access to TS, which at best can only be estimated by summation in conventional IR milk analyzers. The TS information available through PLS from FTIR milk analysis could be quite important, as a variety of multicomponent pricing schemes requiring TS content as a variable are being considered or are in place in various jurisdictions (30).

Now that the price of FTIR spectrometers is dropping, there is a potential for renewed technical innovation in both milk and general dairy product analyses (17). This, in turn, is enhanced through the ability of FTIR spectroscopy, with its advanced data-processing and handling techniques, to make better use of all the information available in the mid IR spectrum. From a broader perspective, such capabilities provide ample opportunity for the advancement of IR analysis to a general quality control tool in the food industry.

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DRUG RESIDUES IN ANIMAL TISSUES

Simultaneous Determination of Sulfonamides in Honey by Liquid Chromatography

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A simple and rapid method using liquid chromatography (LC) for the simultaneous determination of 10 sulfonamides (sulfathiazole, sulfadimethoxine, sulfamonomethoxine, sulfadiazine, sulfamerazine, sulfadimidine [sulfamethazine], sulfamethoxazole, sulfamethoxypyridazine, sulfachloropyridazine, and sulfaquinoxaline) in honey has been developed. Samples were dissolved in 30% sodium chloride and then extracted with dichloromethane. The extracts were cleaned up on a Sep-Pak Florisil cartridge. The LC separation was performed on a LiChrosphere RP-18e column (250 × 4.0 mm id) with 0.05M sodium dihydrogen phosphate–acetonitrile (2 + 1) as the mobile phase; the drugs were detected at 275 nm with 0.04 AUFS. The calibration graphs were rectilinear from 1 to 40 ng for each drug. The recoveries at the level of 0.5 μg/g were 62.1–90.2%, and the detection limits were 0.05 μg/g for each drug. Sulfamonomethoxine was found in 2 samples of domestic products at levels of 0.23 and 0.83 μg/g.

Sulfonamides (SAs) are used for the prevention and treatment of a disease known as American foulbrood (1) in honeybees. The drugs are administered to honeybees as the sodium salt dissolved in sucrose solution. As a result, honey could become contaminated with SAs through this feeding practice. The presence of drug residues in honey is undesirable from the standpoint of food sanitation. Therefore, a simple and reliable method is needed to monitor SAs residues in honey.

Several methods have been developed for the assay of sulfathiazole (STZ) using the colorimetric procedure (2, 3) based on the Bratton-Marshall reaction, thin-layer chromatography (4, 5), gas chromatography (GC) (6), enzyme immunoassay (7), and liquid chromatography (LC) (8–11).

In addition to STZ, sulfamonomethoxine (SMMX), sulfadimethoxine (SDMX), and other SAs have been used in honeybee culture in Japan. However, we have not found any reference to the simultaneous determination of several SAs.

This paper describes a simple and rapid LC method for the simultaneous determination of 10 different sulfonamides that were considered to be possible residues in honey. Sep-Pak Florisil cartridges were used in a cleanup step.

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METHOD

Materials and Reagents

All chemicals were reagent grade or LC grade. Deionized or distilled water was used throughout all experiments.

(a) *Sulfonamides.*—STZ, sulfadiazine (SDZ), sulfamethoxazole (SMXZ), sulfamethoxypyridazine (SMPD), and sulfachloropyridazine (SCPD) (Sigma Chemical Co., St. Louis, MO); sulfamerazine (SMR) and sulfadimidine (SDD; sulfamethazine) (ICN Pharmaceutical Inc., Cleveland, OH); SMMX and SDMX (Daiichi Pharmaceutical Co., Tokyo, Japan); and sulfaquinoxaline (SQ) (Dainippon Pharmaceutical Co., Osaka, Japan).

(b) *Standard solutions.*—(*1*) *Standard stock solutions.*—Dissolve 10.0 mg of each compound in 100 mL methanol. (*2*) *Working standard solutions.*—Dilute stock solution to appropriate concentration with LC mobile phase. (*3*) *Mixed spiking solution.*—Place 2.5 mL of each stock solution in 100 mL volumetric flask, and dilute to volume with LC mobile phase.

(c) *Cleanup cartridges.*—Sep-Pak Florisil (Part No. 51960, Millipore Corp., Chromatography Div., Milford, MA).

(d) *Samples.*—Commercial honey samples were obtained from Saitama prefecture and metropolitan Tokyo.

(e) *Mobile phase.*—Mixture of 0.05M sodium dihydrogen phosphate–acetonitrile (2 + 1), filtered and degassed under vacuum; flow rate, 0.5 mL/min.

Apparatus

(a) *Liquid chromatograph.*—LC-6A solvent delivery system (Shimadzu Corp., Kyoto, Japan), equipped with Rheodyne 7125 loop injector (20 μL loop, Rheodyne Inc., Berkeley, CA), Shimadzu SPD-6A spectrophotometric detector (monitored at 275 nm, range set at 0.04 AUFS), Shimadzu CR-3A integrator and LiChrosphere RP-18e column, 250 × 4.0 mm id, 5 μm

(E. Merck, Darmstadt, Germany) fitted with 4.0 × 4.0 mm guard column of LiChrosphere RP-18e at ambient column temperature.

(b) *Photodiode array detector.*—Model SPD-M6A (Shimadzu) interfaced with Model PC-9801 VX-4 personal computer (NEC Co., Ltd, Tokyo, Japan).

(c) *Evaporator.*—Model N-1 rotary evaporator (Tokyo Rikakikai Co., Ltd, Tokyo, Japan).

(d) *Magnetic mixer.*—Model MH-61 (Yamato Scientific Co., Ltd, Tokyo, Japan).

Extraction and Cleanup

Weigh 5 g honey into 100 mL Erlenmeyer flask. Add 50 mL 30% sodium chloride, and stir until honey dissolves. Transfer dissolved honey to 300 mL separatory funnel and extract with 60 mL dichloromethane. Collect lower layer in 100 mL round-bottom flask and dry with ca 10 g anhydrous sodium sulfate. Apply dichloromethane solution to Sep-Pak Florisil cartridge (prewash cartridge with 10 mL dichloromethane). Wash cartridge with 10 mL acetonitrile–dichloromethane (2 + 8), and elute with 10 mL methanol–dichloromethane (2 + 8). Evaporate to dryness on rotary evaporator at 40°C, and dissolve residue in 1.0 mL LC mobile phase. Filter through Columnguard LCR (pore size, 0.5 μm, Millipore) and inject 10 μL solution into chromatograph.

Calibration Graphs

Prepare standards of each drug at 0.1, 0.2, 0.5, 1.0, 2.0, and 4.0 μg/mL from stock solutions. Inject 10 μL of these solutions into LC system. Obtain calibration graphs by measurement of peak heights.

Recovery Studies

Add 1.0 mL mixed spiking solution (2.5 μg/mL) to 5 g honey. Clean up samples as described above. Inject 10 μL sample solutions into LC system. Measure peak heights and calculate recoveries by comparison with calibration graphs.

Results and Discussion

Chromatographic Conditions

UV detection may be the best available method to detect SAs. The wavelength of detection for STZ in honey has been reported to be 280 nm (10) and 285 nm (11). The 10 SAs were dissolved separately in LC mobile phase and their UV spectra were measured. The maximum UV absorptions of the 10 SAs were found to be in the range of 260–290 nm. On the basis of these results, a wavelength of 275 nm was chosen for the measurement. In the determination of SAs in animal tissues and honey by LC, unlike GC, no derivatization is necessary, and SAs can be determined directly. Although many studies (8–13) have been reported, there have been no reports of the simultaneous determination of SAs in honey.

Many of the LC methods for SAs specify a reversed-phase ODS column that uses the reversed partition mode. Therefore, a study was made of the separation conditions using ODS columns and phosphate buffer–acetonitrile as the mobile phase. Optimum results for detecting SAs were obtained using a LiChrosphere RP-18e (250 × 4.0 mm id) as the column and 0.05M sodium dihydrogen phosphate–acetonitrile (2 + 1) as the mobile phase. Under these conditions, the 10 SAs were successfully separated within 17 min, as shown in Figure 1.

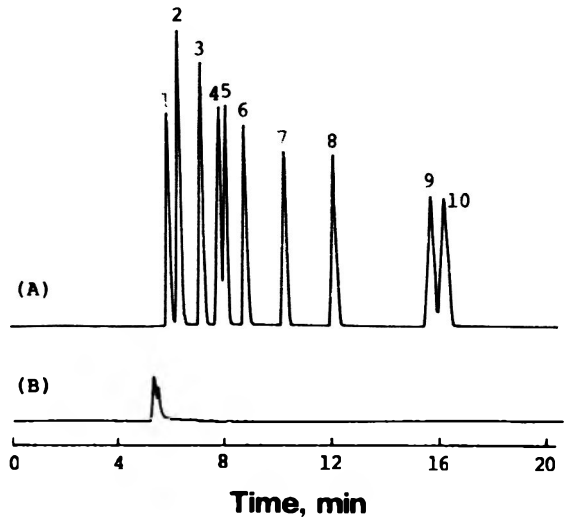

Figure 1. Typical chromatograms of (A) standard mixture of 20 ng (equivalent to 0.4 μg/g) of each drug and (B) honey extract. Peaks: 1 = sulfathiazole (STZ); 2 = sulfadiazine (SDZ); 3 = sulfamerazine (SMR); 4 = sulfamethoxypyridazine (SMPD); 5 = sulfadimidine (SDD); 6 = sulfamonomethoxine (SMMX); 7 = sulfachloropyridazine (SCPD); 8 = sulfamethoxazole (SMXZ); 9 = sulfaquinoxaline (SQ); and 10 = sulfadimethoxine (SDMX).

Cleanup

A universal extraction cleanup is desirable to establish a rapid and widely used determination of residues of antibacterial agents in food. Attempts were made to determine the 10 SAs in honey by the previously proposed method (14, 15) for simultaneous determination of residues of veterinary drugs in fish and meat. The extraction solvent was 0.2% metaphosphoric acid–methanol, and C_{18} cartridges were used. Although this procedure is very simple and rapid, it could not sufficiently remove the interfering substances.

Diaz et al. (11) used a sample solution prepared by dissolving honey in acetonitrile and filtering through a 0.45 μm membrane filter. This method is considered to be unsatisfactory for determining residues of compounds contained in honey, however, because the chromatogram showed a number of peaks due to impurities and the method had a detection limit of 1 μg/g. Barry and MacEachern (9) extracted STZ in honey with acetone and then performed cleanup by liquid-liquid partition. However, the impurities cannot be completely removed by this method. Therefore, we examined a method that combined liquid-liquid partition with the use of dichloromethane, followed by treatment with prepacked cartridges.

Table 1. Effect of concentration of sodium chloride on extraction of sulfonamides

	Rec., %[a]				
Concn, %	STZ	SDZ	SMR	SCPD	Others
0	34.3	77.1	86.6	86.9	>90
2	35.6	78.2	87.0	89.3	>90
5	37.9	80.0	88.5	91.4	>90
10	43.6	84.5	91.3	93.6	>90
20	57.4	89.0	91.7	91.1	>90
30	69.5	91.7	94.0	91.5	>90

[a] Av. of results of 3 replicates.

As Table 1 shows, many SAs were extracted by dichloromethane with a recovery of about 90%. However, the recovery of STZ was extremely low, i.e., 34.3%. We then attempted to increase the recovery by adding NaCl. The increase in the NaCl concentration resulted in a remarkably improved recovery. A 30% NaCl solution (almost saturated) was used for dissolving honey.

The chromatogram of the dichloromethane extract showed peaks due to the impurities. Therefore, we decided to further treat the extract with prepacked cartridges. The effects of the use of Sep-Pak Florisil, silica-gel, and alumina cartridges on the recovery and removal of the impurities were examined. Because the Sep-Pak alumina cartridge showed excessively intense adsorption, recoveries of the 10 SAs were low. On the other hand, the Sep-Pak silica-gel cartridge could not sufficiently remove the impurities, although it achieved a high recovery. The Sep-Pak Florisil cartridges were excellent both for recovery and for removal of the impurities.

When the sample was loaded on the Florisil cartridge and then washed with dichloromethane, the impurities could not be sufficiently removed. Therefore, a dichloromethane–acetonitrile mixture was used as a washing solvent. As a result, the content of the impurities could be lowered with an increase in the acetonitrile content of the washing solvent. When the content of acetonitrile reached 30%, however, SDMX and SDD were lost from the cartridge, as Figure 2 shows. Accordingly, the acetonitrile content was adjusted to 20%.

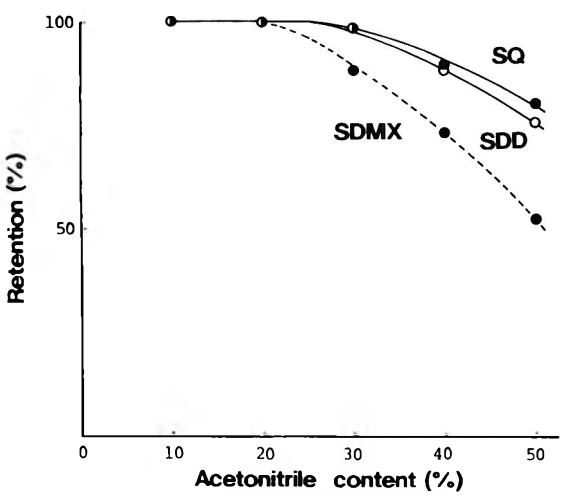

Figure 2. Influence of the acetonitrile content in washing solvent on the retention of sulfonamides on Sep-Pak Florisil cartridge.

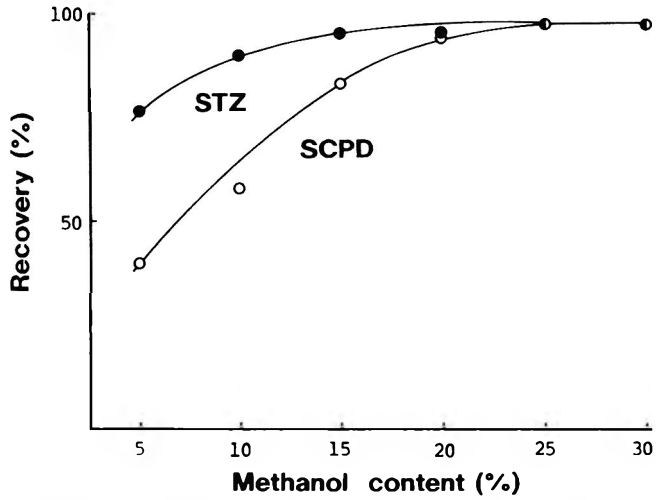

Figure 3. Influence of the methanol content in eluant on the recoveries of sulfonamides from Sep-Pak Florisil cartridge.

When methanol was used to elute SAs from a cartridge, its strong elution capability also resulted in the elution of many impurities. Therefore, dichloromethane–methanol was studied as an eluant. The elution capability increased with an increase in the methanol content of the eluant. Many of the SAs were completely eluted when the methanol content was 10%. However, a methanol content of 20% was required to completely elute STZ and SCPD, as shown in Figure 3. Because a sample solution contaminated with fewer impurities can be obtained by using an eluant with a lower methanol content, dichloromethane–methanol (8 + 2) was used as the eluant.

A typical chromatogram of a honey extract is shown in Figure 1. Cleanup was satisfactory, and no interfering peaks appeared on the chromatogram.

Methanol, ethanol, or amylene was added to the dichloromethane as a stabilizer. When dichloromethane con-

Table 2. Recoveries of sulfonamides from honey[a]

Sulfonamide	Rec., %
Sulfathiazole (STZ)	62.1 ± 3.2
Sulfadiazine (SDZ)	85.6 ± 3.6
Sulfamerazine (SMR)	86.2 ± 1.9
Sulfamethoxypyridazine (SMPD)	87.5 ± 2.3
Sulfadimidine (SDD)	86.0 ± 2.1
Sulfamonomethoxine (SMMX)	90.2 ± 3.0
Sulfachloropyridazine (SCPD)	82.5 ± 0.5
Sulfamethoxazole (SMXZ)	86.4 ± 0.9
Sulfaquinoxaline (SQ)	80.5 ± 3.7
Sulfadimethoxine (SDMX)	85.2 ± 4.1

[a] Values are means ± SD (n = 5). Samples were spiked with each drug at 0.5 μg/g.

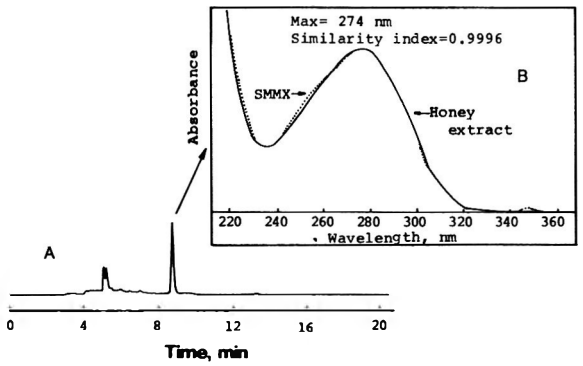

Figure 4. (A) Chromatogram of honey in which 0.23 μg/g sulfamonomethoxine was detected at 275 nm. (B) Normalized spectra of the peak (at 8.7 min) obtained from honey extract (——) and standard sulfamonomethoxine (- - -).

taining methanol or ethanol was used for extracting and washing the cartridge, some portion of the SAs was liberated from the cartridge. Therefore, the dichloromethane containing amylene was used.

Recovery

Linear calibration graphs were obtained from 1 to 40 ng (equivalent to 0.1–4.0 μg/mL) for the 10 SAs. Table 2 summarizes the recoveries of the 10 SAs from honey fortified with 0.5 μg/g. Although the recovery of STZ was low (62.1%), recoveries of other SAs were higher than 80%, with standard deviations within 5%.The detection limits of the method were 0.05 μg/g (signal-to-noise ratio >5) for the 10 of SAs in honey.

Analysis of Commercial Samples

Using the present method, we analyzed 82 commercial samples, including 47 imported products and 35 domestic products. SMMX was found in 2 samples of domestic products at levels of 0.23 and 0.83 μg/g.

In a residue determination, the identification of detected medical substances is needed to confirm an analytical result. A number of GC/mass spectrometric methods developed for confirmation of SAs in animal tissues (16–18) are complicated and time-consuming. We have previously reported a method (19) for the identification of residual SDD in meat using LC provided with a photodiode-array detection. Therefore, this method was applied to the identification of SMMX residues in honey. Figure 4 shows a chromatogram of a honey sample in which SMMX was detected at 0.23 μg/g. The peak component with a retention time of 8.7 min was compared with a standard sample of SMMX. The 2 spectra were almost identical, confirming that the peak component eluting at 8.7 min was SMMX. The similarity index given in Figure 4 represents the similarity of the 2 spectra in terms of numbers. When the similarity index was 0.99 or above, compounds were regarded as identical.

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Determination of Nitroimidazole Metabolites in Swine and Turkey Muscle by Liquid Chromatography

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The method described can determine hydroxy dimetridazole and hydroxy ipronidazole in turkey and swine muscle tissue at levels of 2 ppb. After an initial extraction with ethyl acetate and subsequent evaporation, the samples are solvated in acid and back-extracted. A C_{18} solid-phase extraction is performed after neutralization of the acidic extract. The basis for quantitation was an external standard containing both analytes. After performing 10 recovery curves with blank control tissue spiked at 0, 1, 2, and 4 ppb, the mean recoveries of hydroxy dimetridazole and hydroxy ipronidazole were 61.8 and 64.6%, respectively. Recovery variation was less than 20% for both analytes over all 10 curves. The performance of the extraction method was verified in tissues incurred through the dosing of live animals.

Dimetridazole (DMZ) and ipronidazole (IPR) were found to be effective in the protection of turkey flocks against one of the most frequently occurring parasitic diseases, histomoniasis (1, 2). DMZ was also used in the prevention and treatment of swine dysentery. These compounds were found to be carcinogenic and mutagenic (3). The use of DMZ and IPR for any veterinary application is no longer permitted in the United States (4).

Law et al. (5) described the metabolism of DMZ in turkeys. After 17 h, nearly all of the DMZ is oxidized by a basic metabolic pathway that converts the 2-methyl group to a 2-hydroxymethyl, yielding 2-hydroxymethyl-1-methyl-5-nitroimidazole (DMZOH). Further oxidation of the parent compound can occur to form the carboxylic acid analog or glucuronide conjugate of DMZ. A similar metabolic conversion occurs in which IPR yields 1-methyl-5-nitroimidazole-2-isopropanol (IPROH). The greatest levels of DMZOH and IPROH residues were found in muscle and kidney tissues. The development of accurate and reliable methods of quantitation for IPROH and DMZOH in muscle tissue were, therefore, necessary for food safety.

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A variety of analytical techniques have been used for the determination of the nitroimidazoles and their metabolites in muscle tissue. Craine et al. (6) described a polarographic method for the determination of DMZOH in swine muscle tissue with a sensitivity limit of 2 ppb. Newkirk et al. (7) and Store et al. (8) developed methods for the determination of DMZOH in swine muscle with gas chromatography (GC). Determination of DMZ residues in swine muscle by liquid chromatography (LC) was described by Hobson-Frohock and Reader (9). Carignan et al. (10) described a sensitive method for the analysis of less than 1 ppb DMZ and DMZOH in pork by using LC with electrochemical detection. An enzyme-linked immunosorbent assay for the determination of DMZ and DMZOH in turkey was developed by Stanker (11). The determination of IPR and IPROH in turkey skin and muscle by GC/mass spectrometry with negative ion chemical ionization was described by Garland et al. (12).

The method described can determine both DMZOH and IPROH in swine and turkey muscle tissue at 2 ppb by using LC with UV detection. This straightforward technique can be used to extract 12 samples per day. The alteration of the solid-phase extraction (SPE) columns with stainless steel frits to prevent the introduction of interfering compounds is not required by this method.

Experimental

Generation of Incurred Tissues

Swine.—Feed containing 0.015% DMZ was administered to 6 of 14 market weight hogs for 7.0–7.7 days. Another 6 animals were dosed with 16.26 mg/lb body weight of IPZ per day via stomach tube for 7 days. The remaining hogs were used for the generation of blank control muscle. Hogs treated with DMZ were slaughtered at either 12, 18, or 24 h withdrawal periods. Animals that were administered IPR were slaughtered after 24, 36, or 48 h periods. Each hog had 7.5 lbs of loin and 7.5 lbs of hindquarter tissue excised. Tissue samples were then frozen at −20°C.

Turkey.—Twenty hen turkeys at least 20 weeks old were used in this study. Nine of these turkeys were administered a medicated feed containing 0.015% DMZ for a minimum of

7 days. Another 9 birds were orally dosed with 9 mg/kg body weight of IPR per day for 6.5 days. The 2 remaining turkeys were used for blank control tissue. After either 12, 18, or 24 h, 3 from each set of DMZ- or IPR-dosed turkeys were slaughtered with the thigh and breast muscle tissue excised. Control turkeys were slaughtered with the last withdrawal group to minimize contamination. Tissue samples were then frozen at −20°C.

METHOD

Apparatus

The apparatus listed here may be substituted with an equivalent. No endorsement of these products by the U.S. Department of Agriculture is implied.

(a) *Food chopper.*—Model 4612 (Hobart Corp., Troy, OH).

(b) *Balance.*—Model PE 3600 (Mettler Instruments Co., Hightstown, NJ).

(c) *Centrifuge.*—Model PR-7000 (International Equipment Co., Needham Heights, MA 02194).

(d) *Rotary evaporator.*—Buchler Instruments, Fort Lee, NJ.

(e) *pH Meter.*—Model 701A (Orion Research, Inc., Cambridge, MA 02139).

(f) *SPE columns.*—High capacity C_{18} #7202-07 (J.T. Baker, Inc., Phillipsburg, NJ 08865).

(g) *SPE vacuum manifold.*—Model 7018-00 (J.T. Baker, Inc.).

(h) *N-Evap concentration system.*—Model 111 (Organomation Associates, Inc., South Berlin, MA 01549).

(i) *Liquid chromatograph.*—Model 590 mobile phase pump, Model 721 automated injector, Model 481 UV detector, and Model 730 data module (Waters Chromatography, Div. of Millipore, Milford, MA 01757).

(j) *LC column.*—Hibar RP-18 5 μm, 25 cm (EM Science, Gibbstown, NJ 08027).

Reagents and Materials

(a) *Ethyl acetate.*—Optima grade (Burdick and Jackson, Muskegon, MI 49442).

(b) *Hydrochloric acid, 1M.*—Prepare 1M solution by diluting 83 mL analyzed grade concentrated hydrochloric acid (Baker) with 1 L deionized water.

(c) *Hexane.*—Optima grade (Burdick and Jackson).

(d) *Sodium hydroxide, 5M.*—Dissolve 200 g analyzed grade sodium hydroxide (Baker) in 1 L deionized water.

(e) *Chloroform.*—High purity grade (Burdick and Jackson).

(f) *Deionized water.*—Pass distilled water through a Milli-Q water purification system (Waters).

(g) *Methanol.*—Suitable for LC analysis (Burdick and Jackson).

(h) *Acetonitrile UV.*—Suitable for LC analysis (Burdick and Jackson).

(i) *Phosphoric acid.*—Analyzed grade (Baker).

(j) *Sodium chloride.*—Analyzed grade (Baker).

(k) *Dibasic potassium phosphate.*—Analyzed grade (Baker).

(l) *Monobasic ammonium phosphate.*—LC grade (Fisher).

(m) *Triethylamine.*—99+% purity (Aldrich Chemical Co.).

(n) *Ammonium phosphate buffer.*—Add 5.75 g ammonium phosphate to 1000 mL deionized water. Add 2000 μL triethylamine, and adjust pH to 7.0–7.3 with phosphoric acid.

(o) *Mobile phase.*—0.05M Monobasic ammonium phosphate buffer–acetonitrile (83 + 17). Add 830 mL ammonium phosphate buffer to 170 mL acetonitrile. Pass through a 0.22 μm filter before use.

Standards and Fortification Solutions

(a) *Hydroxy DMZ.*—Merck and Co., Inc., Rahway, NJ 07065.

(b) *Hydroxy IPR.*—Hoffman LaRoche, Inc., Nutley, NJ 07110.

(c) *DMZOH stock solution (500 μg/mL).*—Dissolve 25 mg DMZOH in 50 mL ethyl acetate.

(d) *IPROH stock solution (500 μg/mL).*—Dissolve 25 mg IPROH in 50 mL ethyl acetate.

(e) *Mixed fortification solution (5 μg/mL or 5 ng/μL).*—Add 1 mL each stock solution to a 100 mL volumetric flask. Dilute to volume with ethyl acetate.

(**Note**: A 2 ppb fortified sample is made by spiking 50 g turkey or swine muscle with 20 μL mixed fortification solution.)

Tissue Homogenization and Extraction

Sample homogenization.—Pass turkey or swine muscle through a meat grinder once (plate size 5/32 in.). Store in a freezer (temperature −5°C) until 50 g muscle tissue is to be taken from the ground sample.

Sample extraction.—Allow homogenized muscle tissue samples to thaw for ca 30 min. Add 50 g thawed turkey or swine muscle tissue to 10 g dibasic potassium phosphate and 10 g NaCl in a 250 mL centrifuge tube. Prepare a recovery sample by spiking a blank tissue with 2 ppb DMZOH and IPROH. Add 100 mL ethyl acetate to each sample, and shake manually for 1 min. Centrifuge 5 min at 1500 rpm ($600 \times g$). Transfer supernatant to a 500 mL round-bottom flask. Add another 100 mL ethyl acetate, and repeat shaking and centrifugation steps. Combine ethyl acetate extracts in the 500 mL round-bottom flask.

Evaporate the combined extracts to an oily residue by using a rotary evaporator with the water bath set at 50–55°C. Add 2 mL 1M HCl and 2.5 mL ethyl acetate to each sample and swirl contents. Decant the mixture into a 125 mL separatory funnel containing 15 mL hexane. Repeat the addition of 1M HCl and ethyl acetate to the flask containing the oily residue. Transfer this mixture to the separatory funnel containing hexane. Gently shake the separatory funnel for 1 min, and let contents equilibrate for at least 20 min. Collect the aqueous layer in a scintillation vial and neutralize with 5M NaOH or 1M HCl to a pH of 4.8–5.2.

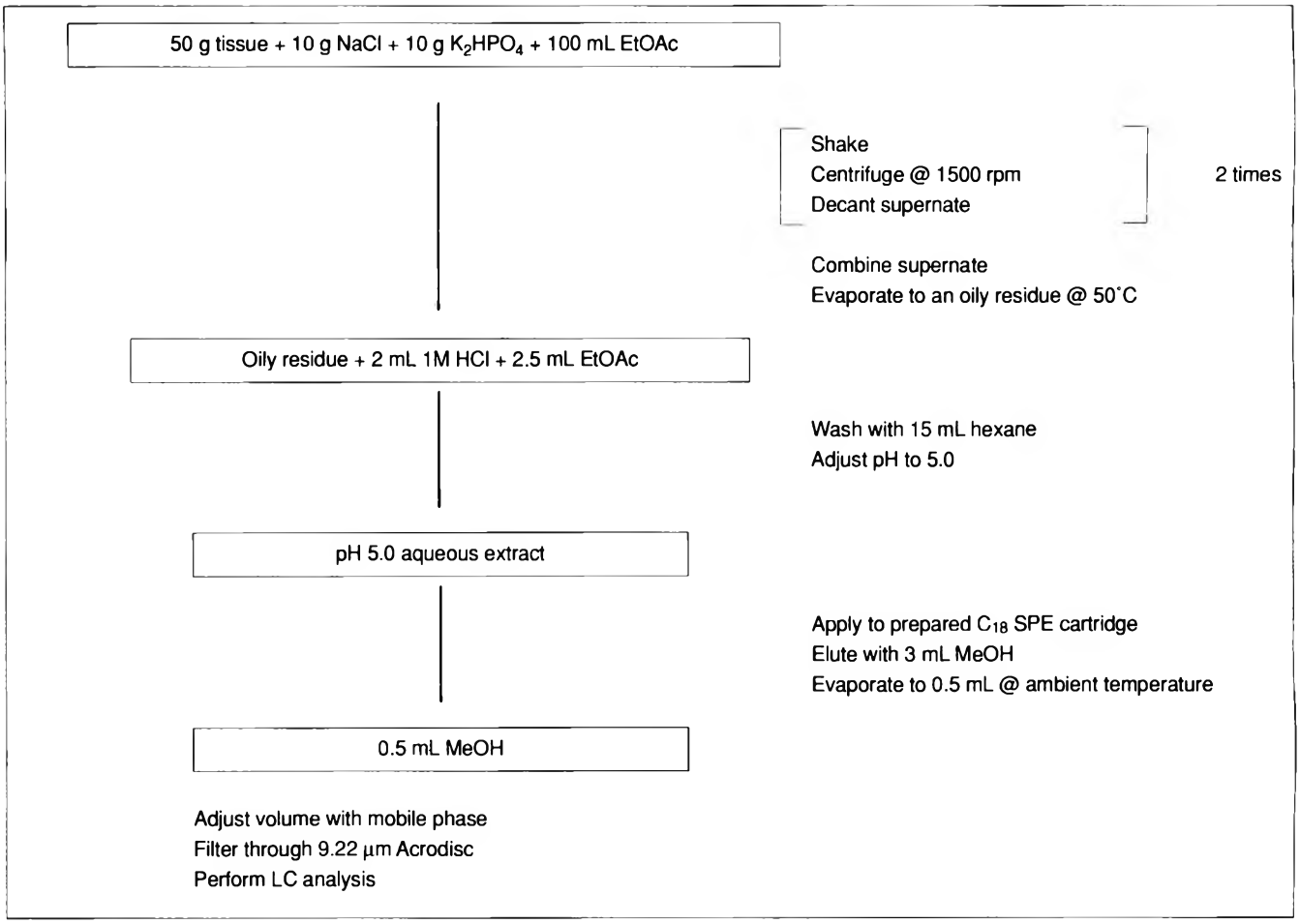

Figure 1. Flow chart of the extraction method.

Prepare the high capacity C_{18} SPE columns by washing each with 10 mL methanol, 10 mL chloroform, 10 mL methanol, and 20 mL water. Allow each solvent to be drawn through each SPE column by using a vacuum equal to 2 in. mercury. Do not let the columns dry while performing these solvent washes. Add the neutralized extracts to the SPE columns, and allow these samples to flow through by gravity. Aspirate the columns to dryness. Add 3 mL methanol to each SPE column, and allow this solvent to pass through by using a vacuum equal to 1 in. mercury. Collect effluent and transfer it to a 10 mL graduated conical tube. Evaporate the effluent to 0.5 mL by using an N-Evap, and reconstitute to 1 mL with mobile phase. Transfer the extract to a syringe with a 0.22 µm Acrodisc filter attached, and force the reconstituted sample into a 1 mL autosampler vial. A flow chart of the extraction method is shown in Figure 1.

LC conditions.—Operate the LC system with a flow rate of 0.6 mL/min. Inject 30 µL sample onto the analytical column. Set UV detector at a wavelength of 325 nm, with a sensitivity of 0.005 AUFS. Set chart speed at 0.40 cm/min.

Calculations

Screening.—Calculate DMZOH and IPROH content based on manually measured peak heights as follows:

$$\text{ng analyte on-column} = P_{samp}/(P_{std} \times R)$$

where, assuming the same volume of sample and external standard are injected, P_{samp} = the peak height of analyte in the sample; P_{std} = the peak height of DMZOH or IPROH in the external standard; and R = the percent recovery of analyte. Convert from ng on-column of analyte to ppb in sample as follows:

$$\text{ppb of analyte in muscle} = (\text{ng analyte on-column} \times V_s/V_i)/W$$

where V_s = the total volume of extract; V_i = the volume of sample injected; and W = the weight of the sample of muscle taken.

Quantitation

Calculate quantitative values by generating a regression curve of response of recovered analyte vs concentration at the 1, 2, and 4 ppb levels. A linear curve described by the equation $y = mx + b$ results, where y = response value of the analyte, m = slope of the regression curve, x = concentration of analyte in sample, and b = y-intercept of the regression curve. Solving the equation describing a linear curve for x yields $x = (y - b)/m$. Determine the concentration of analyte in a sample (x) after obtaining values for m, b, and y. Plot a recovery curve or perform a

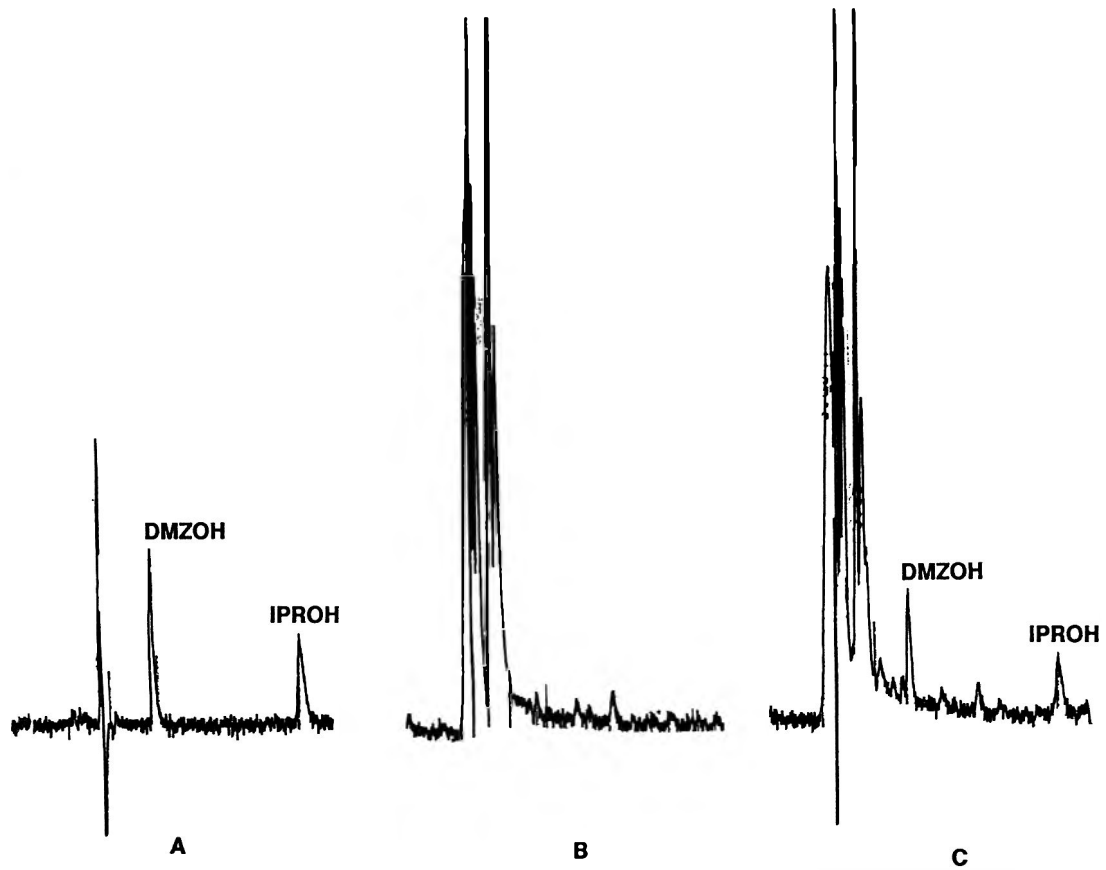

Figure 2. Typical chromatograms of (a) 2 ppb mixed external standard, (b) blank turkey muscle, and (c) 2 ppb recovery of DMZOH and IPROH from turkey muscle.

Table 1. Recoveries of DMZOH and IPROH from turkey and swine muscle

	DMZOH				IPROH			
Day	4 ppb	2 ppb	1 ppb	Correlation coefficient	4 ppb	2 ppb	1 ppb	Correlation coefficient
				Turkey				
1	61.5	60.0	56.4	0.9999	62.5	65.0	60.0	0.9992
2	68.6	65.7	57.1	0.9999	62.5	60.0	60.0	0.9998
3	62.2	67.6	59.4	0.9971	63.2	73.7	73.6	0.9960
4	64.1	64.1	66.7	0.9999	65.0	70.0	60.0	0.9971
Mean	64.1	64.4	59.9	—	63.3	67.2	63.4	—
SD	3.19	3.21	4.71	—	1.18	5.96	6.80	—
CV, %	5.0	5.0	7.9	—	1.9	8.9	10.7	—
				Swine				
1	55.0	55.0	67.0	0.9982	56.0	56.0	63.0	0.9996
2	59.7	66.7	55.6	0.9947	60.5	63.1	63.1	0.9997
3	72.8	62.8	57.1	0.9989	76.3	68.4	63.1	0.9994
4	61.5	60.0	56.4	0.9999	62.5	60.0	80.0	0.9943
5	55.7	60.0	51.4	0.9971	52.5	55.0	50.0	0.9989
6	64.9	67.6	70.3	0.9999	71.0	78.9	84.2	0.9992
Mean	61.6	62.0	59.6	—	63.1	63.4	67.2	—
SD	6.61	4.71	7.33	—	9.01	8.80	12.64	—
CV, %	10.7	7.6	12.3	—	14.3	13.9	18.8	—

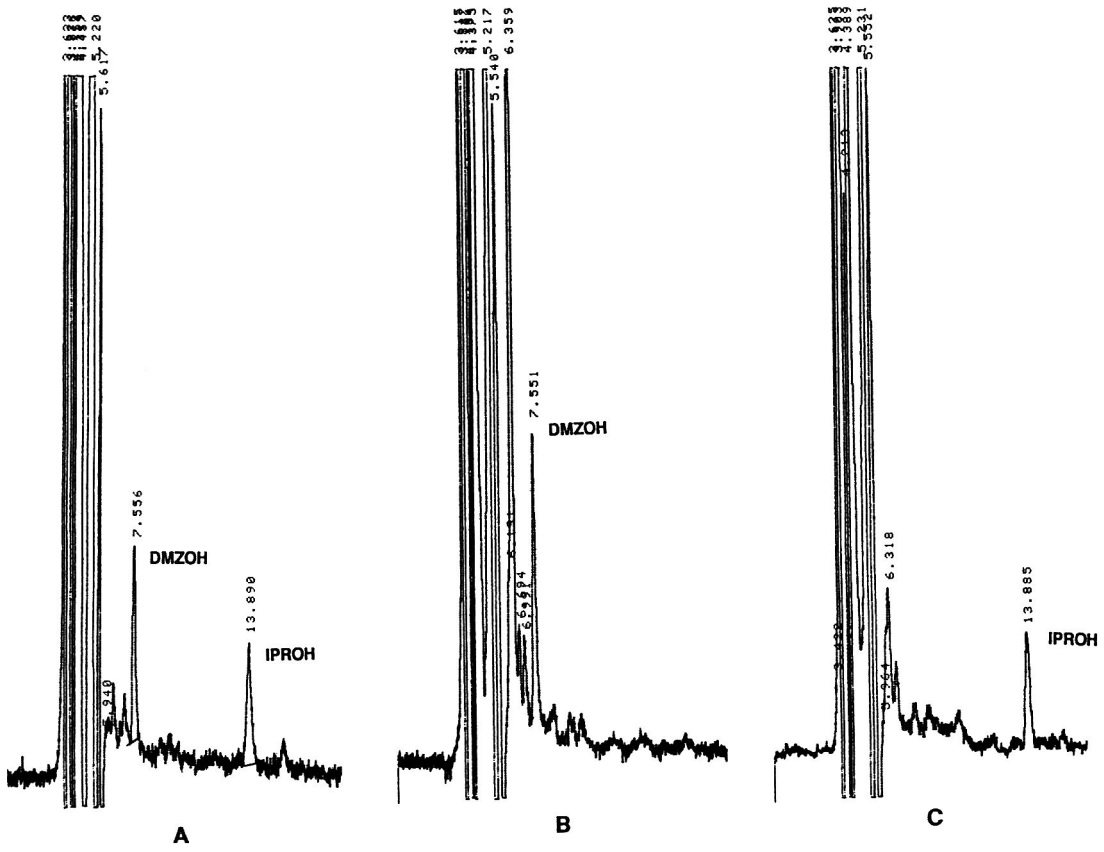

Figure 3. Chromatograms from incurred tissue of (a) 2 ppb recovery of DMZOH and IPROH from swine muscle, (b) swine muscle incurred with 2.9 ppb DMZOH, and (c) swine muscle incurred with 1.8 ppb IPROH.

linear regression calculation to obtain values for the slope (m) and the y-intercept (b). The variable y represents the response value of the analyte found in the sample to be quantitated.

Results and Discussion

The extraction scheme described here is designed to remove nonpolar, highly polar, or ionic matrix components. Small modifications in this method caused significant increases in recovery. The organic layer in the liquid-liquid back-extraction described in the method section consists of 25% ethyl acetate and 75% hexane. During the initial phases of the development of this extraction method, ethyl acetate was used as the partitioning solvent. Use of hexane in the liquid-liquid extraction enhanced the recovery of DMZOH and IPROH. When more than 40% hexane in ethyl acetate was used in the liquid-liquid partition step, a matrix compound appeared to elute at the retention time of IPROH. Studies of the UV spectra of IPROH and its interferant indicated that a shift in wavelength from 315 to 325 nm greatly decreased the absorptivity of the interferant relative to that of IPROH. Incorporation of this wavelength shift along with the use of hexane in the liquid-liquid extraction yielded significant increases in analyte recovery. The sensitivity of this method was thereby enhanced. Extraction efficiency was also increased by incorporating ethyl acetate into the solvation mixture that was applied to the oily residue that remained after rotary evaporation.

Six swine and 4 turkey tissue recovery curves were generated. The swine and turkey tissues used came from large composite samples of muscle tissue. Extraction variabilities in terms of recovery were more readily detected if sample-to-sample variations could be minimized. No interfering components were found to elute at the retention times of DMZOH or IPROH (Figure 2). Each set of control tissues was spiked at 0, 1, 2, and 4 ppb of both analytes. Recoveries were measured by peak height comparison with a 2 ppb external standard. Recovery coefficients of variation of <20% were obtained for all spiked levels of DMZOH and IPROH in swine and turkey muscle. The correlation coefficients for all recovery curves were greater than 0.9940 (Table 1).

The determination of DMZOH and IPROH in tissues incurred at the 2 ppb level has been performed (Figure 3). The results of an incurred tissue study for both turkey and swine are shown (Tables 2 and 3). Although wide variations exist in the levels reported for certain tissue and analyte combinations, these results could not be traced to any discrepancy in dosing or any unusual physical characteristics of the treated animals.

Table 2. Incurred tissue study results in swine

DMZOH			IPROH		
Sample No.	Loin, ppb[a]	Hindquarter, ppb[a]	Sample No.	Loin, ppb[a]	Hindquarter, ppb[a]
Control	0.0	0.0	Control	0.0	0.0
12-133-1[b]	3.1	4.5	24-3-1	1.7	4.0
12-133-2	2.9	7.0	24-3-2	1.7	3.4
12-136-1	8.7	6.4	24-6-1	12.1	14.5
12-136-2	8.8	10.7	24-6-2	14.5	7.5
18-134-1	TR[c]	1.2	36-2-1	1.5	1.9
18-134-2	1.3	2.5	36-2-2	1.4	1.6
18-137-1	1.3	1.7	36-5-1	TR	TR
18-137-2	TR	1.6	36-5-2	TR	TR
24-135-1	0.0	0.0	48-1-1	TR	TR
24-135-2	0.0	0.0	48-1-2	TR	TR
24-138-1	0.0	0.0	48-4-1	TR	TR
24-138-3	0.0	0.0	48-4-2	TR	TR

[a] All the levels shown represent the average of 2 replicate extractions.
[b] The first 2 characters of the sample number indicate the number of hours of withdrawal before slaughter.
[c] TR Indicates less than 1.0 ppb present.

Muscle tissues incurred with IPROH and DMZOH were exhaustively extracted to determine the efficiency of the initial extraction of incurred analytes from tissue. The initial extraction of a swine muscle tissue incurred with DMZOH solvated 650 ng of this analyte, while a second extraction removed 125 ng DMZOH. An initial extraction of a swine muscle tissue incurred with IPROH solvated 18 μg of this analyte, while a second extraction of the same tissue removed 2.3 μg IPROH. On the basis of these results, 80–90% of these metabolites were solvated by using the initial extraction procedure described above.

This method has not been tested for interferences from other drug metabolites; however, no false-positive results were reported after the analysis of 500 turkey and swine muscle samples from a wide variety of sources in and outside the United States. Parent DMZ has been identified as an interference at the retention time of IPROH when part per million levels of DMZOH are present in incurred muscle tissue.

Conclusion

We have developed a method of detection at the 2 ppb level for both IPROH and DMZOH for regulatory purposes. A recovery, a tissue blank, and 10 samples can be prepared daily for LC analysis by using this rapid extraction procedure.

Table 3. Incurred tissue study results in turkey

DMZOH			IPROH		
Sample No.	Thigh, ppb[a]	Breast, ppb[a]	Sample No.	Thigh, ppb[a]	Breast, ppb[a]
Control	0.0	0.0	Control	0.0	0.0
12-444[b]	2.0	1.3	12-424	680	645
12-472	15.0	31.0	12-453	505	515
12-476	2.3	1.6	12-475	2300	2300
18-442	1.7	3.3	18-450	1050	1550
18-470	2.4	1.7	18-462	60	60
18-482	1.4	1.2	18-479	1030	1150
24-428	1.1	1.1	24-441	610	595
24-446	1.7	1.7	24-454	365	195
24-471	TR[c]	1.2	24-480	240	330

[a] All the levels shown represent the average of 2 replicate extractions.
[b] The first 2 characters of the sample number indicate the number of hours of withdrawal before slaughter.
[c] TR indicates less than 1.0 ppb present.

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DRUG RESIDUES IN ANIMAL TISSUES

Liquid Chromatographic Determination of Methylene Blue and Its Metabolites in Milk

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A method is described for the liquid chromatographic (LC) determination of trace levels of methylene blue (MB) and its metabolites in milk. The cleanup involves protein precipitation with acetonitrile, extraction into chloroform of MB and its metabolites (azure A [AZA], azure B [AZB], and azure C [AZC]), extraction by chloroform of thionin at pH 10, and solid-phase extraction on a disposable carboxylic acid column. LC separation and quantitation are performed with an isocratic acetonitrile–acetate buffer mobile phase on a cyano column. Average recoveries (5–20 ppb levels) of MB, AZA, AZB, and thionin from fortified milk were 83.2, 60.0, 84.4, and 22.5%, respectively. Some incurred MB metabolites in milk are bound organically to a fraction of the milk substrate, whereas others are free demethylated forms of MB. Analysis of milk collected 8 h after administration of MB contained the following average levels (n = 6) of free MB and metabolites: MB, 31.0 ppb; AZA, 21.3 ppb; and AZB, 54.1 ppb. Depletion of free MB and metabolites AZA and AZB from milk after administration of MB to lactating cows occurred in less than 40 h. Metabolites of MB that formed complexes with organic compounds were hydrolyzed in part to the free forms. The leuco form of MB could not be verified in incurred residues in milk.

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Methylene blue (MB) is a veterinary drug that can be used as an antiseptic and disinfectant or in the treatment of cyanide and nitrate poisoning (methemoglobinemia) in cattle and sheep. Because MB and its metabolites are considered mutagens (1), the potential for incurred residues of MB in milk and tissues requires methodology for their determination. A literature search revealed few analytical methods for detecting these compounds in milk and tissues. Our principal objective was to develop a method to separate and measure MB and its metabolites in milk.

In lactating cows, the permeability of the blood–milk barrier to MB was reported to be rapid, and MB is depleted from the blood more quickly than from milk (2). The mechanism of transfer apparently involves the reduced (leuco) form of MB; however, there is little information relative to the demethylation of MB in lactating cows except that it is believed to occur (3). DiSanto and Wagner (3, 4) reported that MB compounds are reduced in vivo to the leuco form in animals and that both forms are found in urine. The presence of MB and the colorless

leucomethylene blue (LMB) metabolites in urine was determined spectrophotometrically (3). Because the LMB compounds are stabilized by the salts in the urine and do not partition into organic solvents, MB is partitioned and determined. Warming an acidified urine solution before extraction frees the LMB, which oxidizes rapidly to the colored MB. The colored MB is subsequently determined.

Few chromatographic methods are reported in the literature for residues of MB, the demethylated azures A (AZA), B (AZB), and C (AZC), and for thionin in foods. Chromatography of these compounds is, for the most part, found in general separation procedures for dyes. Thin-layer chromatography (TLC) and silica column chromatography were used to separate these compounds in a study designed to identify impurities in commercial dyes (5). In another method, TLC and liquid chromatography (LC) showed that MB does not degrade in ophthalmic preparations (6). Gagliardi et al. (7) identified cosmetic dyes by LC, using a C_8 reversed-phase column with a perchlorate ion-pairing mobile phase. Van Liedekerke et al. (8) reported the LC separation of quaternary ammonium compounds, using a polystyrene–divinylbenzene column that improved peak symmetry. Roybal et al. (1) used an LC-cyano column with electrochemical detection to measure MB at residue levels. That method (1) was initially used in this study; however, a variable wavelength detector was substituted for the electrochemical detector, because the sample matrix fouled the electrochemical detector cell. We report here a method for the LC determination of MB and its metabolites as residues in milk.

Experimental

Apparatus

(**a**) *Liquid chromatograph.*—Model 6000 pump (Waters Associates, Milford, MA 01757), Model 1050 automatic sample injector (Hewlett-Packard Co., Avondale, PA 19311-0900), and Model GM 970 monochromator detector equipped with tungsten lamp (ABI, Kratos Div., Ramsey, NJ 07446). Set wavelengths at maximum absorption for each compound as follows: MB, 660 nm; AZA, 620 nm; AZB, 635 nm; thionin, 603 nm; and mixtures of AZA and AZB, 627 nm. Set flow rate at 1.0 mL/min.

(**b**) *Integrator.*—HP Model 3396A integrator/recorder (Hewlett-Packard Co.)

(**c**) *LC column.*—Alltech Econosil/CN column, 5 μm particle size, 250 × 4.6 mm id (Cat. No. 605CN, [new Cat. No. 60138], Alltech Associates/Applied Science, Deerfield, IL 60015).

(**d**) *Rotary evaporator.*—Buchi Model R-110 with ice trap (Brinkmann Instruments, Inc., Westbury, NY 11590).

(**e**) *Evaporator.*—N-Evap Model 111 (Organomation Associates, Inc., South Berlin, MA 01549-0159).

(**f**) *Syringe.*—Glass, 2 mL (Becton Dickinson Microbiology Systems, Cockeysville, MD 21030).

(**g**) *Flask.*—Pear-shaped, 100 mL, 24/40 *T* (Cat. No. K-608700, Kontes Co., Vineland, NJ 08360).

(**h**) *Filter.*—Disposable Millex-FH4, 4 mm diameter, 0.5 μm (pore size) polytetrafluoroethylene (PTFE)/polypropylene (Cat. No. SJFHL04NS, Millipore Corp., Bedford, MA 01730).

(**i**) *Tubes.*—Falcon graduated 50 mL centrifuge tubes (Cat. No. 2098, Becton Dickinson, Lincoln Park, NJ 07035). Disposable test tube 13 × 100 mm.

(**j**) *Autosampler vial.*—Polypropylene, 0.1 mL (Cat. No. 225190, Wheaton Scientific, Millville, NJ 08332).

Reagents

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

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

(**c**) *Acetate buffer.*—Prepare by adjusting 0.1 M sodium acetate solution (8.2 g/1000 mL) to pH 4.5 with acetic acid (ca 8 mL).

(**d**) *Mobile phase.*—Acetonitrile–acetate buffer (1 + 1).

(**e**) *Eluting solution.*—1N HCl–methanol (1 + 99).

(**f**) *Diluting solution.*—Water–acetonitrile (60 + 40).

(**g**) *Carboxylic acid (CBA) column.*—Bond Elut LRC, disposable (Cat. No. LR15304, Analytichem International, Harbor City, CA 90710). Suitability test: Condition column with 4.0 mL acetonitrile; let solvent just enter column bed by using pressure or vacuum. Add 0.100 mL MB diluted standard to 4.0 mL acetonitrile and transfer solution to CBA column. Draw solution into column bed and wash column with another 4.0 mL acetonitrile. Elute MB with 2 mL eluting solution (**e**). The blue color should (*1*) be retained on top of the column after the acetonitrile wash, (*2*) migrate in a thin band with eluting solvent, and (*3*) completely elute before 2 mL eluant has passed through column.

(**h**) *Stock standards.*—Prepare stock standards of 100 μg/mL in methanol of the following: methylene blue (Cat. No. 42800, United States Pharmacopeial Convention, Inc., Rockville, MD 20852); AZA and AZB (Cat. Nos. C8689 and C8683, respectively, Eastman Kodak Co., Rochester, NY 14650); AZC and thionin (Cat. Nos. 24, 218-7 and 86,134-0, respectively, Aldrich Chemical Co., Milwaukee, WI, 53233). Adjust standard weights for label purity.

(**i**) *Diluted individual standards.*—Dilute 1.00 mL aliquot of each stock standard to 100.0 mL with water (1.00 μg/mL). *Individual working standards.*—Transfer 0.100 mL aliquot of each diluted standard to separate injection vial and add 0.400 mL diluting solution (**f**) to vial (0.200 μg/mL).

(**j**) *Diluted mixed standard.*—Transfer 1.00 mL aliquot of MB stock standard into 100 mL volumetric flask. Add appropriate aliquots of the other stock standards to produce chromatographic peaks of approximately the same height as that of the MB peak. Dilute to 100.0 mL with water. *Working mixed standard.*—Transfer 0.100 mL aliquot of diluted mixed standard to injection vial and add 0.400 mL diluting solution (**f**). Use this standard for calculation of total MB plus metabolites.

Milk Samples

Control milk and milk containing incurred residues were supplied by the Division of Veterinary Medical Research, Center for Veterinary Medicine. MB (8.8 mg/kg of body weight)

Table 1. Recovery of methylene blue and its metabolites from fortified milk

Analyte	*n*	Added, ppb	Av. rec. ± SD, ppb	Rec., %	CV, %
MB	6	5.24	4.1 ± 0.55	78.2	13.2
	6	10.5	8.8 ± 0.49	83.8	5.50
	6	20.9	18.4 ± 1.04	88.0	5.65
AZA	6	10.2	4.1 ± 0.72	40.2	17.3
	6	20.3	16.2 ± 0.423	79.8	2.63
AZB	5	9.65	7.4 ± 0.55	76.7	7.39
	5	19.3	17.8 ± 1.36	92.2	7.65
Thionin[a]	4	18.6	4.19 ± 2.2	22.5	53.3

[a] Extracted from pH 10 aqueous phase.

was administered in a 1% (w/v) isotonic saline solution as a single dose to a lactating cow by slow intravenous infusion via the jugular vein. Control milk was taken from the animal before dosing. The post-treatment milk was collected at 8, 24, 32, 48, 56, and 72 h after administration of MB. All collections were quickly frozen before shipment. The frozen milk was then broken into pieces small enough to be placed into quart jars for storage in a −70°C freezer. A portion of the frozen milk was thawed, and assay amounts were measured into centrifuge tubes and frozen until needed.

Acetonitrile Extraction

To 10 mL milk in 50 mL centrifuge tube, add 40 mL acetonitrile. Stopper tube, shake tube vigorously for 10 s, and centrifuge 10 min at 3500 rpm. Transfer 45 mL aliquot to 125 mL separatory funnel, taking care to prevent transfer of solids, which would cause emulsion problems in the partition step.

Partition Cleanup

Add 5 mL 0.1M NaCl and 25 mL chloroform to separatory funnel containing acetonitrile extract. Extract MB and metabolites by gently inverting funnel ca 30 times in 1 min to minimize emulsion formation. Let mixture stand until emulsion breaks. Drain lower layer into 100 mL pear-shaped flask. Add another 25 mL portion of chloroform to separatory funnel and repeat extraction, combining lower layer with contents of flask. Take care to prevent transfer of emulsion to flask.

For thionin assay, add 1N NaOH to remaining contents of separatory funnel to adjust pH to 10. Extract thionin with two 25 mL portions of chloroform. After each extraction in this assay, drain lower layer into 100 mL pear-shaped flask set aside for thionin fraction.

Evaporate contents of both flasks to dryness by using rotary evaporator and water bath set at 40°C. Add 4 mL acetonitrile to each flask and combine contents. Again evaporate to dryness to remove all water. Dissolve residue in 4 mL acetonitrile.

Column Cleanup

Condition CBA column by passing 3–4 mL acetonitrile through column. Transfer contents of flask to column, and let solution pass through at a rate of 3–6 mL/min by adjusting pressure or vacuum.

Wash flask and column with two 2 mL portions of acetonitrile; let each portion enter column bed before adding the next. Collect column effluent without applying pressure and discard. Elute MB and metabolites with two 1 mL portions of eluting solution followed by 1 mL methanol. Collect eluate in 13 × 100 mm disposable test tube.

Evaporate eluate to dryness with nitrogen stream directed at contents of tube placed in water bath set at 40°C. Dissolve residue in 0.200 mL acetonitrile–water (60 + 40), and vigorously mix contents of tube on vortex mixer. Transfer contents of tube to 2 mL syringe fitted with 0.5 μm filter. Collect filtrate in autosampler vial.

Liquid Chromatography

Inject 30 μL filtrate into liquid chromatograph. Bracket injections of filtrates from a group of samples with 30 μL injections of appropriate working standard.

Determine concentration of individual compounds at wavelength setting of maximum absorption for each. Calculate concentration of each individual compound in milk as follows:

$$\text{ppb} = (A_{SPL}/A_{STD}) \times C_{STD} \times (0.200\ \text{mL}/10.0\ \text{g})$$

where A_{SPL} = area of peak of interest in chromatogram of injected filtrate, A_{STD} = peak area in chromatogram of standard, and C_{STD} = concentration of standard injected (ng/mL).

Determine concentration of total MB plus metabolites at wavelength setting of 627 nm. Calculate concentration of total MB plus metabolites in milk as follows:

$$\text{ppb} = (A_{SPL}/A_{STD}) \times 5 \times C_{STD} \times (0.200\ \text{mL}/10.0\ \text{g})$$

where A_{SPL} = total area of 5 peaks of interest in chromatogram of injected filtrate, A_{STD} = total area of 5 peaks in chromatogram of mixed standard, and C_{STD} = concentration of MB standard injected (ng/mL).

Results and Discussion

Emulsion formation during the partition step, caused by the presence of solids, reduced recoveries and the speed of analy-

Table 2. Levels of incurred residues in milk, based on individual assays of MB, AZA, and AZB, vs levels based on total peak area

Post-treatment collection, h	Analyte	*n*	Av. found, ppb	CV, %
	From individual assays			
8	MB	6	31.0 ± 2.9	9.49
	AZA	6	21.3 ± 2.1	9.65
	AZB	6	54.1 ± 5.3	9.88
	All	—	106 ± 6.3[a]	5.92
	From total peak area			
8	All	5	176 ± 11.0	6.25
24	All	5	10.2 ± 1.0	10.1
32	All	3	5.3 ± 2.1	40
48	All	2	2.3	—
Control	All	1	2.4	—

[a] Sum of levels from individual assays of MB, AZA, and AZB.

sis. Emulsions were avoided by taking an aliquot of the solid-free acetonitrile extract instead of decanting the solution from the centrifuge tube. Additionally, a CBA solid-phase extraction column from each lot was tested to ensure uniformity between lots relative to the retention characteristics of MB.

Recovery studies (Table 1) were conducted with portions of control milk that were thawed, fortified with the appropriate standard, and assayed at the optimum visible wavelength for each compound. The amount recovered and the coefficient of variation (CV) were adequate for all the metabolites except thionin. Thionin could be extracted into chloroform from the deproteinated fraction only by adjusting the pH to 10; however, thionin recoveries were low and variable. Data for the recovery of AZC from milk were inconclusive because of the low purity of the available standard.

Applying the proposed method to the incurred residues in milk from an MB-dosed cow by assaying for individual compounds gave reasonable CVs (Table 2). However, because this approach was too time-consuming, we chose to measure the total peak area of the free MB compounds and to compare this response to that of a mixed standard containing approximately equal amounts of MB, AZA, AZB, AZC, and thionin. Pure standards of AZA, AZB, and AZC were not available. The content of the mixed standard was based on the calculated area ratios of the impurities. Higher values were obtained for the assays based on total peak area than for the totals of the assays for individual compounds; the CVs were approximately the same.

It became obvious during this study that MB had undergone complex chemical changes resulting not only in demethylated metabolites, i.e., the azures and thionin, but also in MB–organic complexes that complicated the cleanup procedure. The free metabolites present in milk, however, are easily separated and quantified by LC (Figure 1). Attempts to liberate complexed MB with hydrochloric acid resulted in only the partial

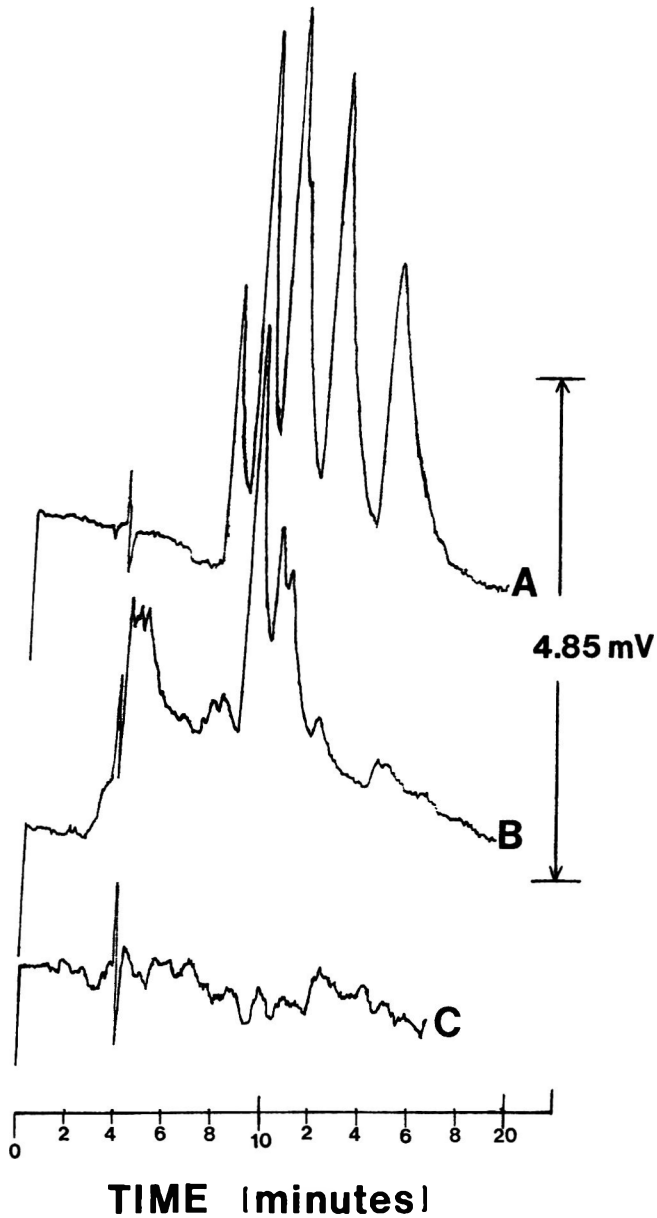

Figure 1. Chromatograms of (A) mixed standard containing, in order of elution, thionin, AZC, AZA, AZB, and MB; (B) incurred residues in milk collected 8 h post-treatment; and (C) control milk at higher attenuation. The detector wavelength was set at 627 nm.

liberation of all forms of oxidized MB. Because the contribution of complexed MB was not included, total MB is not accounted for in the amounts reported for milk (Table 2). These MB complexes were not studied in sufficient detail to note their total effect. We are presently investigating the means to completely release complexed MB for this analysis as well as the chromatography of the complex.

Although DiSanto and Wagner (3) reported a stable leuco form of MB in urine, we found no evidence to indicate the presence of incurred LMB in milk. The aqueous fraction that was left after the free MB compounds were extracted was subjected to an acid treatment in an attempt to liberate additional colored compounds. Heating the acidified solution while si-

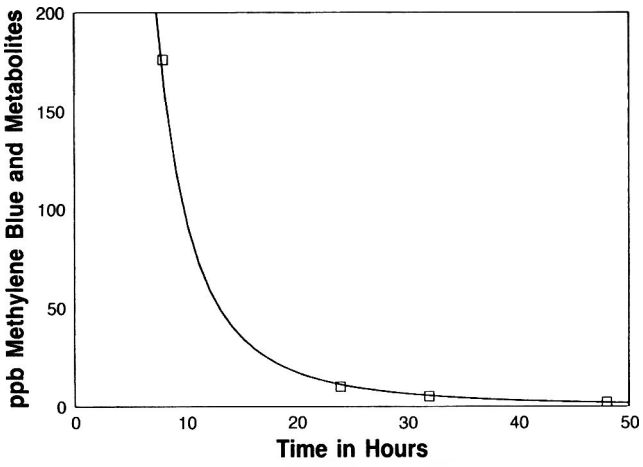

Figure 2. Depletion of incurred residues of free MB and its metabolites in milk collected post-treatment, as measured by total peak area.

multaneously bubbling air through it did not result in the formation of additional colored compounds. Therefore, if LMB compounds were present in the milk, they were bound in such a way as to prevent the liberation of LMB by our treatment.

Incurred MB is rapidly depleted from milk to a level equal to that of apparent MB in control milk, as shown in Figure 2. This was obvious from a visual inspection of the post-treatment frozen milk, which revealed a greenish cast that diminished with each milk collection but could still be observed in the 32 h collection. The MB level in the 32 h milk collection corresponds to the limit of detection for free MB (2.5 ppb) and metabolites. Complexed MB may account for some of the color in the frozen milk, but this could not be determined because no method presently exists for assaying complexed MB. The methodology presented here for MB and its metabolites in milk indicates that additional research is required to develop a method for the assay of complexed MB. Overall, the method is suitable for the determination of free MB (5, 10, and 20 ppb), AZB (10–20 ppb), and AZA (20 ppb); however, modifications to the method are required for assaying AZC and thionin. The presence of multiple metabolites and the unavailability of pure standards complicate method development for these compounds. The analysis of milk for incurred residues indicated a rapid depletion of MB and oxidized MB, with the highest concentration found in the 8 h post-treatment collection. For assays of MB, AZA, and AZB with calculation using the quantitative sum of the individual peak areas, the method is consistent, with an acceptable standard deviation and CV (Table 2). Thus, it may be most appropriate to indicate the presence of MB as the total of MB plus all detectable metabolites.

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DRUGS

Liquid Chromatographic Determination of Alprazolam and Related Impurities in the Drug Substance

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A liquid chromatographic method was developed for the determination of alprazolam and 5 known related compounds in drug raw materials. The method is based on a 3 μm cyano column (100 × 4.6 mm), a mobile phase of 0.01% phosphoric acid–acetonitrile–methanol (6 + 3 + 1), a flow rate of 1.5 mL/min, and detection at 230 nm. The method resolves the related compounds from the drug and from each other. The limit of quantitation for the impurities in drug raw material is less than 0.1%. Impurity levels in 4 drug raw materials were between 0.01 and 0.41%. The corresponding mean assay values ranged from 98.3 to 100.6%.

The *United States Pharmacopeia* (USP) monograph for 8-chloro-1-methyl-6-phenyl-4H-s-triazolo [4,3-a][1,4]-benzodiazepine (alprazolam), a sedative, requires determination of chromatographic purity by gas (GC) and thin-layer chromatography (TLC) (1). Method A, based on GC, sets a 1.0% limit on the total of all impurities; Method B, based on TLC, limits single impurities to 0.3% and the total of all impurities to 1.0%. Normal-phase liquid chromatography (LC) is used for drug raw material assay, and reversed-phase LC is used for tablets. There are no monographs for alprazolam in the *British Pharmacopoeia*. Other LC methods have been reported for the determination of alprazolam in formulations (2, 3), body fluids (4–6), and postmortem specimens (7).

Alprazolam-related compounds possibly present in the drug raw material were proposed from known routes of synthesis and degradation. Samples of as many of these compounds as were available were obtained. They are listed under *Reagents*. None of the above methods was sufficiently selective and sensitive for the quantitation of the available related compounds. A method for this purpose is described in this paper.

Experimental

Reagents

(a) *Related compounds.*—The structures and names of alprazolam and 5 related compounds are shown in Figure 1. Compounds II, III, and V are synthetic intermediates; IV is a potential side reaction product; and VI is a synthetic intermediate and hydrolysis product. Related compounds were obtained from Profarmaco (II, III, IV, and V) and Aldrich Chemical Co., Inc., Milwaukee, WI (VI).

(b) *Acetonitrile and methanol.*—LC grade (J.T. Baker, Phillipsburg, NJ).

(c) *Phosphoric acid, 85%.*—LC grade (Fisher Scientific, Fair Lawn, NJ).

(d) *Deionized water.*—Prepared with a Sybron/Barnstead system.

Apparatus

(a) *LC system.*—Model 5060 (Varian, Sunnyvale, CA) equipped with a Model SP8780 XR autosampler with a 10 μL loop injector (Spectra-Physics Analytical, San Jose, CA), a Model UV-100 UV detector set at 230 nm (Varian), and a Vista 402 data station (Varian). A No. 118963, 100 × 4.6 mm, 3 μm, nitrile column (Spherisorb, Chromatography Sciences Co.) was used at ambient temperature with a mobile-phase flow rate of 1.5 mL/min.

(b) *Centrifuge.*—Model K (International Equipment Co., Needham Heights, MA)

(c) *UV–vis spectrophotometer.*—DMS 90 (Varian) connected to a HP 85 computer with a plotter and disk drive.

(d) *Wrist action shaker.*—Eberbach Corp., Ann Arbor, MI.

(e) *Horizontal action shaker.*—Model 75 (Burrel Corp., Pittsburgh, PA).

(f) *Balance.*—Model AK 160 (Mettler Instrument Corp., Hightstown, NJ).

LC Method

(**Note**: Do not dry alprazolam standards or samples. Use all solutions within 4 h of preparation.)

Mobile phase.—Add 50 mL deionized water to a 100 mL volumetric flask and then transfer 12.0 mL LC grade H_3PO_4 (85% by weight) to the flask. Dilute to volume with deionized water. Transfer 1.0 mL of this solution to a 1 L volumetric flask and dilute to volume with deionized water. Transfer 300 mL acetonitrile and 100 mL methanol to a 1 L volumetric flask and dilute to volume with the phosphoric acid solution. Filter through a 0.45 μm Nylon-66 filter.

Solutions.—Prepare all solutions in acetonitrile–methanol–water (3 + 1 + 6). (*1*) *System suitability solution.*—0.01 mg/mL Alprazolam and 7-chloro-5-phenyl-1,3-dihydro-2H-

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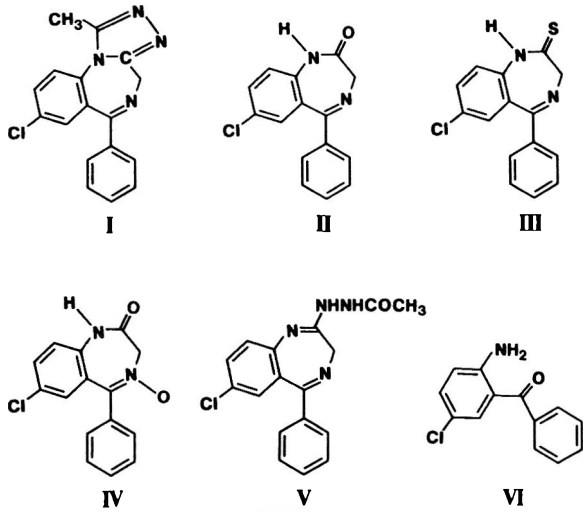

Figure 1. Structures of alprazolam and related compounds: (I) 8-chloro-1-methyl-6-phenyl-4H-s-triazolo [4,3-a][1,4]benzodiazepine (alprazolam); (II) 7-chloro -5-phenyl-1,3-dihydro-2H-[1,4]benzodiazepin-2-one; (III) 7-chloro-5-phenyl-1,3-dihydro-2H-[1,4]benzodiazepin-2-thione; (IV) 7-chloro-5-phenyl-1,3-dihydro-2H-[1,4]benzodiazepin-2-one *N*-oxide; (V) 7-chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin -2-acetyl-hydrazine; and (VI) 2-amino-5-chlorobenzophenone.

[1,4]benzodiazepin-2-one (II). (*2*) *Related compounds standard solution.*—0.001 mg/mL Alprazolam and 0.001 mg/mL 2-amino-5-chlorobenzophenone (VI). (*3*) *Related compounds test solution.*—0.5 mg/mL Alprazolam. (*4*) *Assay standard solution.*—0.01 mg/mL Alprazolam. (*5*) *Assay test solution.*—0.01 mg/mL Alprazolam.

System suitability.—Inject a 10 µL aliquot of the resolution solution into the LC system. The efficiency of the column, calculated by using the alprazolam peak, is not less than 30 000 plates/m, the resolution is more than 5, and the tailing factor is 1.5 or less, all calculated according to USP procedures. The retention times of alprazolam and II are ca 2.5 and 5.9 min, respectively. Six injections of the related compounds standard solution gave a coefficient of variation (CV) less than 5%. Five injections of the assay standard solution give a CV less than 1%.

Procedures.—(*1*) *Related compounds.*—Inject separately 10 µL of the related compound standard and related compound test solutions into the chromatograph and record the peak area responses for 30 min. Calculate the percentage of each impurity, except VI, from the following equation:

$$\% \text{ Impurity} = 100 \times (A_i/A_s) \times (C_s/C_u)$$

where A_i is the peak area response of each impurity, A_s is the peak response of alprazolam in the related compounds standard solution, C_s is the concentration of alprazolam in the related compounds standard solution, and C_u is the concentration of alprazolam in the related compounds test solution. To calculate the concentration of VI, A_s is the peak response of VI in the related compounds standard solution, and C_s is the concentration of VI in related compounds standard solution.

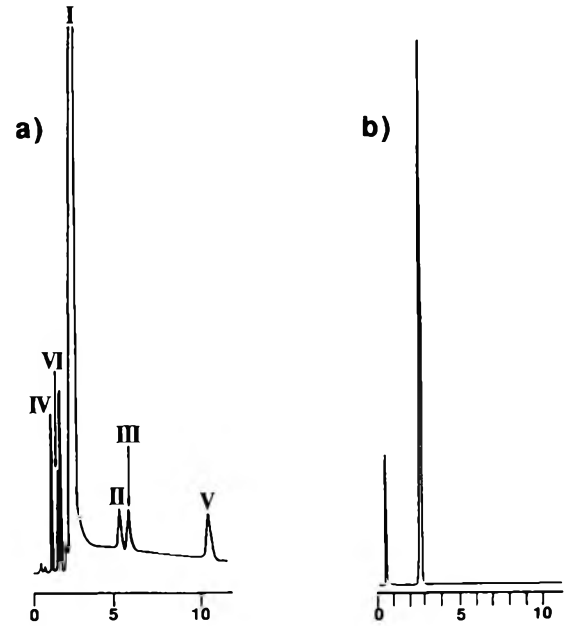

Figure 2. Liquid chromatograms. (a) Alprazolam and related compounds; the amounts on-column were alprazolam (I), 5.24 µg; II, 0.004 µg (0.08%); III, 0.0125 µg (0.24%); IV, 0.007 µg (0.13%); V, 0.022 µg (0.42%); and VI, 0.006 µg (0.11%). A high alprazolam loading, resulting in peak tailing, was necessary to achieve quantitation at the 0.05% level. The peak at RRT 0.73 is an unidentified impurity in alprazolam Sample B1. (b) Alprazolam at 0.1 µg on-column, which corresponds to the assay concentration of 0.01 mg/mL.

Drug assay.—Inject separately 10 µL assay standard solution and assay test solution into the chromatograph and record the peak area responses. Calculate the percentage of alprazolam from the following equation:

$$\% \text{ Alprazolam} = 100 \times (A_u/A_s) \times (C_s/C_u)$$

where A_u and A_s are the areas of alprazolam in the assay test solution and assay standard solution, respectively, and C_s and C_u are the concentrations of alprazolam in the assay standard solution and assay test solution, respectively.

Discussion

USP chromatographic purity Method A, previously evaluated for triazolam, gave poor peak shape and poor resolution of some compounds. The method was not further evaluated. Method B, a TLC method, and the LC assay method were evaluated, the LC method for its intended purpose and for the determination of related compounds.

The quantitation of related substances by TLC was inaccurate because of diffusion of the spots. In addition, by comparison with the LC method presented in this paper, several unknown impurities were not resolved from the drug spot.

The USP LC assay method was evaluated for the determination of related compounds. Compounds II, III, and IV were inadequately resolved from the solvent front, and Compound V was poorly resolved from the drug.

Table 1. UV characteristics of alprazolam and related compounds

Compound	Maxima, nm	Absorption at 230 nm	Relative absorption	Concn, µg/mL
I	221	0.741	1.00	5.0
II	201, 228	0.543	0.92	4.0
III	201, 301	0.439	0.37	8.0
IV	201, 236, 307	0.90	0.76	8.0
V	201, 260	0.778	0.52	10.0
VI	200, 236	0.763	0.64	8.0

A representative chromatogram showing the resolution of the available related compounds from alprazolam (Lot B1) and from each other is given in Figure 2. The UV and LC characteristics of alprazolam and the related compounds are given in Tables 1 and 2, respectively. The method provides for quantitation of VI against a standard of VI because of the toxicity of this compound and the need, in any standard based upon this method, to quantitate it accurately at low levels. Other impurities are quantitated against an alprazolam standard.

Results

Related compounds.—Four drug raw material samples were available for analysis at the time this work was done. They are coded by letter to indicate manufacturer and by number to distinguish between samples from the same manufacturer. Impurities found in drug raw materials are given in Table 3.

Drug content.—Alprazolam raw materials were assayed in triplicate. Mean results, with the CVs in parentheses, were A, 98.3% (0.56); B1, 100.1% (1.17); and B3, 99.7% (0.21). Sample B2 was used as a reference standard.

Method Characteristics

Linearity and sensitivity.—The response to alprazolam was linear from 2.5 to 170 ng on-column, and the response to the related compounds was linear from 2.5 to 35 ng on-column, with correlation coefficients greater than 0.998, except for V, which was 0.946. The limit of quantitation, taken as the lowest concentration to give a CV of less than 10%, was 0.05% (0.1% for V).

System precision.—Six replicate injections of 2 alprazolam solutions (0.00527 and 0.012654 mg/mL) gave CVs of 0.59 and 0.23%, respectively.

Method precision.—Raw material B1, assayed in triplicate on 5 successive days, gave a mean of 99.5% alprazolam, with a CV of 1.2%.

Solution stability.—A solution of alprazolam showed no extraneous peaks after 2 h in a volumetric flask under short or long UV light. The solution was also stable for 24 h under laboratory conditions. In solution, V slowly transforms into a mixture of alprazolam and II. After 0.5, 1, 2, and 18 h, 1.6, 3, 6, and 51% of Compound V were transformed. The method calls for the analysis of related substances to be performed within 4 h of solution preparation so that V, if present, will be detected.

Ruggedness.—Two columns were used during the course of this work (Serial No. 118962 and 118963). Both gave similar retention times for all compounds. Changes of 3% or less in mobile-phase composition had virtually no effect on the order of elution or separation of the related substances from the drug. Phosphoric acid is essential for the resolution of the related compounds. Buffer solutions of ammonium, sodium, or potassium phosphate at the same pH resulted in a loss of resolution of some impurities from the drug peak.

References

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Table 2. LC characteristics of alprazolam and related compounds

Compound	RRT[a]	Slope[b]	Intercept[c]	R^2	Linearity range[d]	Limit, %[e]	Response[f]
I	1.0	1156	1548	0.998	2.49–24.9	0.05	1.00
	—	965	–2582	0.999	52.7–168.7	—	—
II	2.37	1320	–200	0.999	2.17–30.36	0.04	1.14
III	2.98	620	–564	0.999	2.62–36.71	0.05	0.54
IV	0.44	1041	799	0.999	2.58–36.16	0.05	0.90
V	5.0	1899	–5779	0.946	4.38–21.88	0.09	1.64
VI	0.68	904	–138	0.999	2.28–32.0	0.04	0.78

[a] Retention time relative to alprazolam at 2.5 min.
[b] Area counts per nanograms on-column.
[c] Area counts.
[d] Nanograms on-column.
[e] On the basis of the lowest concentration that integrates with a coefficient of variation of 10% or less, relative to 5 µg alprazolam on-column.
[f] LC response relative to that of alprazolam.

Table 3. Related compounds in alprazolam raw materials (%)[a]

RRT[b]	A[c]	B1	B2	B3
0.66	—	0.01	0.01	0.03
0.71	—	0.40	—	0.04
0.76	0.03	—	—	—
0.87	0.07	—	—	—
0.93	0.13	—	—	—
Total	0.23	0.41	0.01	0.07

[a] Quantitation was by comparison to an external alprazolam standard corresponding to 0.2% impurity.
[b] Retention time relative to alprazolam at 2.5 min.
[c] Results are the means of 3 determinations except for B1, which is the mean of 6 determinations.

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DRUGS

Gas Chromatographic Methods for Doxepin Isomers, Related Compounds, and Organic Volatile Impurities in Raw Materials and Doxepin Isomers in Capsules

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Health and Welfare Canada, Health Protection Branch, Bureau of Drug Research, Tunney's Pasture, Ottawa, ON, K1A 0L2, Canada

Gas chromatographic methods have been developed for the determination of doxepin as the sum of the resolved *E* and *Z* isomers, 4 doxepin-related compounds, and organic volatile impurities in drug raw material. Minimum quantifiable amounts of the related compounds in raw materials are less than 0.05%. A method for assay of doxepin, as the total of the *E* and *Z* isomers, in capsules was also developed; relative standard deviation is less than 1% for the drug assay. Eleven raw material and 18 capsule samples were examined. No drug-related compounds or *U.S. Pharmacopeia*- or *European Pharmacopeia*-limited organic volatile impurities were detected in any of the samples.

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Doxepin hydrochloride, an antidepressant, is a 1:5 mixture of *cis* and *trans* (*Z* and *E*) isomers. Structures and chemical names of the drug and several related compounds are given in Figure 1. Doxepin may be prepared (1) by transesterification of phenol with phthalide to 2-phenoxymethylbenzoic acid, ring closure by an intermediate acid chloride in the presence of a Lewis acid to doxepinone, and reaction with a nucleophile (2), such as a Grignard reagent, to doxepinol. Dehydration of doxepinol under acidic condition yields doxepin.

Both the *U.S. Pharmacopeia* (USP) (3) and *British Pharmacopeia* (4) contain monographs for doxepin and various formulations. Neither pharmacopeia limits drug related impurities, although the USP limits certain organic volatile impurities (5). The USP gas chromatographic (GC) method for doxepin assay was evaluated as a possible method for related compounds. Only the peaks due to Compounds III and V were well-resolved. Compound II, an immediate precursor of the drug in some syntheses, did not elute, and Compound V almost merged with the solvent peak. The USP proposes to change the assay

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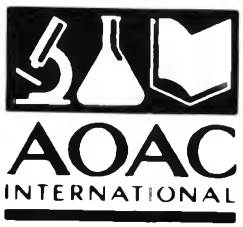

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procedure from GC to liquid chromatography (LC) (6). There are numerous methods in the literature for the determination of doxepin in biological fluids (7). The present report describes capillary GC methods for determination of doxepin isomers, related compounds, and organic volatile impurities in raw material and for the isomer content of capsules.

Experimental

Apparatus

(a) *GC system.*—Model HP 5890A gas chromatograph equipped with flame ionization detector, Model 7673 autosampler, Model 3396A integrator, and Model 9122C disk drive (Hewlett-Packard Co., Palo Alto, CA).

(b) *GC/Fourier transform IR (FTIR) system.*—Model LSC-2000 purge-and-trap system (Tekmar) with capillary interface to Model 5890A gas chromatograph (Hewlett-Packard) and Model FTS 40 Fourier transform infrared detector with GC/C 32 accessory (Digilab).

(c) *Column.*—5% Phenyl dimethylpolysiloxane bonded-phase, 15 m × 0.53 mm, 1.5 μm. A 30 m column was used for determination of organic volatile impurities (DB-5, J & W Scientific, Folsom, CA).

Reagents

(a) *Chemicals.*—LC grade chloroform and reagent grade acetic anhydride (J.T. Baker, Phillipsburg, NJ); LC grade acetic acid (Calendon Laboratories, Georgetown, ON, Canada); and

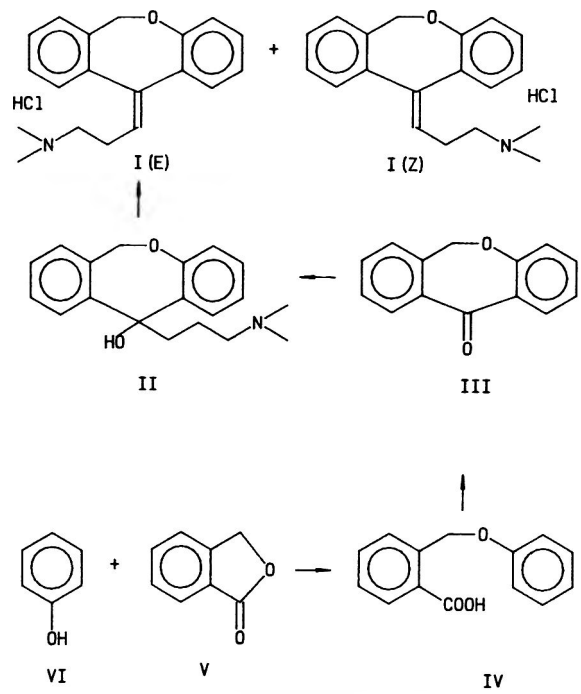

Figure 1. Structures of doxepin and related compounds: I, doxepin hydrochloride (1-propanamine-3-dibenz[*b,e*]oxepin-11(6H)ylidene-*N,N*-dimethylhydrochlo r-ide); II, doxepinol (1-propanamine-3-dibenz[*b,e*]oxepin-11-ol-*N,N*-dimethylhydrochloride); III, doxepinone (6,11-dihydrodibenz[*b,e*]oxepin-11-one); IV, 2-phenoxy methylbenzoic acid; V, phthalide; and VI, phenol.

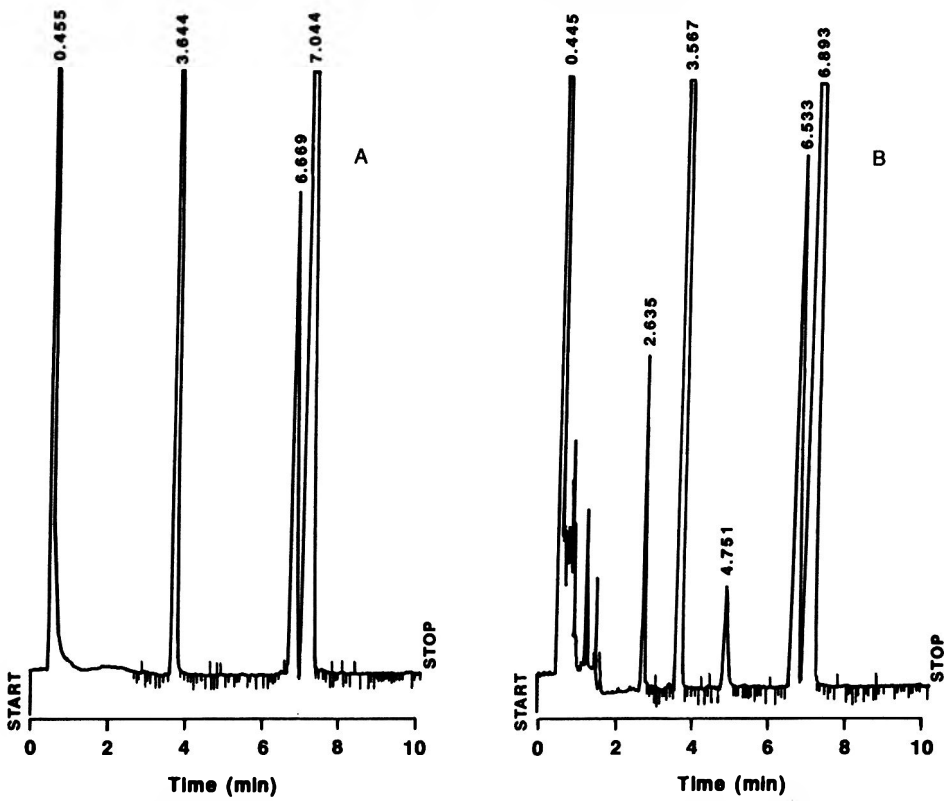

Figure 2. (A) Gas chromatogram of doxepin raw material A1 and chlorpheniramine maleate (3.6 min); the *Z* and *E* isomers elute at 6.6 and 7.0 min, respectively. (B) Capsule sample G3; the peaks are as follows: chlorpheniramine maleate, 3.6 min; excipient fatty acids, 2.6 and 4.7 min; *Z* isomer, 6.5 min; and *E* isomer, 6.8 min.

Table 1. Linearity of (*Z*)- and (*E*)-doxepin and related compounds

Compound	Relative retention[a]	Slope, area/ng[b]	Intercept, area counts	Range, ng
(*Z*)-Doxepin[c]	0.98	104	186	100–1000
(*E*)-Doxepin[c]	1.00	109	102	100–1000
II	1.07	256	14	0.5–9.0
III	0.75	312	80	0.5–9.0
V	0.48	307	49	0.5–9.0
VI	0.30	226	54	0.5–10

[a] Retention time relative to (*E*)-doxepin at 15.5 min.
[b] R^2 was greater than 0.999 in all cases.
[c] Determined on the purified maleate salts.

perchloric acid, 0.1N (Fisher Scientific, Fair Lawn, NJ). Local tap water is sufficiently free of volatile organic compounds to be used without treatment for direct injection analysis. Before use in the purge-and-trap apparatus, with a concentration ratio of 5000:1, the water was purged 1 h with nitrogen. Other chemicals were reagent grade or better.

(**b**) *Doxepin-related compounds.*—Compounds II, III, and IV and drug raw material (SmithKline Chemicals, Conshohocken, PA; Plantex, Netanya, Israel; Teva Pharmaceuticals, Petah Tiqva, Israel; Secifarma, Milan, Italy; and Yodogawa Pharmaceuticals, Osaka, Japan); (*E*)- and (*Z*)-doxepin maleate (Plantex); phenol and phthalide (Aldrich, Milwaukee, WI); and chlorpheniramine maleate (Sigma, St Louis, MO). IR and NMR spectra of these compounds corresponded with their structures. Doxepin hydrochloride capsule products were obtained locally.

(**c**) *Internal standard solution.*—1.0 mg/mL chlorpheniramine maleate in methanol, accurately known, prepared fresh daily.

Assay and Isomer Distribution of Doxepin in Raw Material and Capsules

Standard solution.—USP Reference Standard doxepin hydrochloride, 1.0 mg/mL, was accurately prepared in internal standard solution. USP Reference Standard doxepin is labeled to show the percentages of the *E* and *Z* isomers.

Raw material test solution.—A 1.0 mg/mL doxepin hydrochloride solution was accurately prepared in internal standard.

Capsule test solution.—Empty 5 capsules into a suitable volumetric flask, add internal standard solution, and shake 30 min on a wrist action shaker to dissolve. Dilute accurately with internal standard solution to a final concentration of ca 1 mg/mL doxepin. Mix well, remove a 3 mL aliquot, and centrifuge 5 min.

GC operating conditions.—Set the injection port and detector at 250°C and the oven at 220°C. Use the split mode with a column and split to flows of 8.5 and 333 mL/min. Use helium for the carrier gas.

System suitability test.—Make 6 replicate injections of the standard solution. The resolution of the *E* and *Z* (small peak) isomers is not less than 1.4, and the relative standard deviation (RSD) of the peak responses due to the *Z* isomer is less than 1%.

Procedure.—Inject separately 1.0 µL test and standard solutions, and record responses of the peaks due to chlorpheniramine maleate and the *Z* and *E* isomers. Elution times are ca 4, 7, and 7.5 min, respectively. Calculate the concentration of each doxepin isomer in the test solution as follows:

$$C \times (R_U/R_S)$$

where C is the concentration of the isomer in the standard solution, R_U is the ratio of the peak response of that isomer to the response of chlorpheniramine maleate obtained from the test solution, and R_S is the corresponding ratio of peak responses obtained from the standard solution. The total doxepin concentration is the sum of the concentrations of the *E* and *Z* isomers.

Table 2. Reproducibility of the assay method for raw material and capsules (%)

Day	A2	G5, 50 mg	G11, 100 mg
1	99.6	98.2	102.8
2	101.0	98.3	102.7
3	99.9	99.9	101.8
4	100.7	98.8	104.3
5	99.8	99.4	102.8
Mean	100.2	98.9	102.9
RSD	0.61	0.73	0.87

Related Compounds in Doxepin Hydrochloride Raw Material

Standard solution.—A 0.005 mg/mL doxepinone in methanol solution, accurately known.

Test solution.—A 1.0 mg/mL doxepin hydrochloride in methanol solution, accurately known.

GC operating conditions.—Set the injection port and detector temperatures at 250°C. Operate the injection port in the splitless mode for 0.5 min, then split the helium carrier gas to flows of 8.5 and 333 mL/min. Program the oven as follows: 2 min at 40°C, 25°C/min to 200°C, 5°C/min to 260°C, and hold 10 min.

Procedure.—Inject separately 1.0 µL standard and test solutions into the chromatograph and record peak responses. Calculate the percentage of each impurity peak by comparison with the response of the doxepinone peak in the standard solution.

Table 3. Assay and isomer content in doxepin hydrochloride raw materials (%)[a]

Code	Assay	RSD	*Z* Isomer	RSD	*E* Isomer	RSD
A1	100.8	0.58	15.2	1.92	85.5	0.35
A2	99.6	0.64	14.3	0.83	85.3	0.62
A3	100.4	0.48	15.5	1.24	84.9	0.41
A4	100.5	0.52	15.7	1.40	84.7	0.45
A5	99.8	0.34	15.1	0.61	84.7	0.53
A6	100.3	0.63	15.4	3.50	84.8	0.09
B1	100.2	0.67	15.9	1.02	84.3	0.61
B2	100.2	0.73	15.3	3.28	84.9	0.30
B3	100.4	0.69	13.7	4.56	86.7	0.21
C1	99.6	0.16	15.2	0.74	84.4	0.05
D1	99.5	0.79	15.3	1.97	84.2	0.58

[a] All determinations in triplicate. USP doxepin hydrochloride Reference Standard Lot G-1 was used as a standard.

Organic Volatile Impurities in Doxepin Hydrochloride

Test solution.—Accurately weigh ca 17 mg doxepin hydrochloride into a 1.7 mL vial, crimp close, and add 1.7 mL water.

Standard solution.—With a 10 µL syringe, accurately measure 10 mg dichloromethane, benzene, trichloroethylene, and 1,4-dioxane and 5 mg chloroform into a 1 L volumetric flask filled with organic-free water and dissolve. Transfer 1.0 mL of this solution to a 10 mL volumetric flask, and dilute to volume with water to give final concentrations of 0.5 µg/mL chloroform and 1.0 µg/mL of the other compounds.

GC operating conditions.—Set the helium carrier gas flow rate to ca 6 mL/min, the nitrogen makeup gas to 30 mL/min, the air flow to 400 mL/min, and the hydrogen flow to 35 mL/min. Use the splitless mode with a purge flow of ca 80 mL/min, activated at 0.5 min. Temperature program for the column oven: 35°C for 4 min, 5°C/min to 50°C, 20°C/min to 100°C, and hold 15 min.

Procedure.—Separately inject 1.0 µL water (blank), standard, and test solutions into the chromatograph. If there are any peaks in the test solution whose retention times are similar to those in the standard or with area counts greater that 10 000 (ca 1000 ppm or 0.1% of the drug), continue with the following identification procedure.

Identification.—Dissolve 200 mg doxepin hydrochloride in 5.0 mL nitrogen purged water, and transfer to the purge-and-trap accessory. Purge the sample 4 min with helium at 40 mL/min. Rapidly heat to 80°C then purge a further 11 min, dry the trap by purging 2 min with helium, and desorb the sample 4 min at 180°C into the capillary inlet at −150°C. Heat the capillary inlet to 220°C for 0.75 min to transfer the sample to the chromatograph. The chromatographic flow and temperature conditions are the same as above. Set the temperature of the FTIR light pipe and transfer lines at 200°C, and set the resolution at 8 cm^{-1}. Collect data at 4 scans/s. Identify the unknown impurity by comparison of its IR spectrum with spectra in the FTIR vapor-phase library.

Results and Discussion

In the following discussion, raw material and capsule samples are coded by letter to represent the manufacturer, followed by a number to distinguish between lots from the same manufacturer.

Assay

Chromatograms showing resolution of the *E* and *Z* isomers from each other and the internal standard in raw materials and a capsule product are presented in Figure 2. System response, given in Table 1, was linear from 4 to 40 ng (7 points) on column (100–1000 ng before splitting). The day-to-day reproducibility of the assay method was determined by analyses done on 5 consecutive days (Table 2). The RSD (n = 5) was less than 0.7% for drug raw material and less than 1% for 25 and 100 mg capsules. The RSD of 6 consecutive injections was 0.65% for the peak responses and less than 0.07% for the retention times.

During method development, we noticed that the contents of some capsules were inhomogeneous, consisting of fine and course particles, the latter 1–2 mm in diameter. These phases were sieve separated and analyzed for doxepin. The mean assay value of the fine particles was 60.8% of label claim and that of the larger particles was 373.4% of label claim. To avoid inhomogeneous test samples, the method requires the test solution to be made up by dissolving the entire contents of 5 capsules.

Results of the triplicate analysis of 11 raw material samples from 4 manufacturers are presented in Table 3. Total drug content was between 99.5 and 100.8%, with RSD from 0.16 to 0.79%. The *E* isomer range was from 84.2 to 86.7%, with RSD from 0.05 to 0.62%. Capsule assay results in Table 4, representing 3 manufacturers, ranged from 94.5 to 106.2%, with the highest RSD at 3.5%.

Related Compounds

Figure 3 shows the resolution of doxepin-related compounds from the drug and each other. System response was linear from 0.5 to ca 9 ng on-column (4 points) for Compounds II, III, V, and VI, with the square of the correlation coefficient greater than 0.999 (Table 1). The lower limit of quantitation for these compounds in doxepin is less than 0.05%, and the limit of detection is approximately 0.01%. The limit of quantitation was taken to be the concentration that, on 5 injections, gave an

Table 4. Assay and isomer content in doxepin capsules (%)[a]

Code	Dose, mg	Assay	RSD	*Z* Isomer	RSD	*E* Isomer	RSD
E1	10	98.7	1.13	15.0	0.98	83.7	1.16
E2	100	100.4	3.28	14.8	4.53	85.6	3.08
E3	150	100.3	1.76	14.7	0.73	85.6	1.97
F1	10	100.7	1.52	14.2	1.61	86.4	1.50
F2	100	100.2	1.14	14.2	4.05	86.0	0.88
G1	10	99.4	1.75	14.0	2.19	85.4	1.90
G2	10	94.6	3.22	14.4	3.11	80.2	3.24
G3	25	101.3	0.83	15.5	1.34	85.4	0.77
G4	25	97.8	3.43	14.8	3.31	89.9	3.52
G5	50	94.8	1.60	14.5	2.05	80.3	1.52
G6	50	100.6	1.88	14.4	1.97	86.2	1.86
G7	50	99.5	0.45	13.9	4.65	85.6	1.21
G8	50	94.5	1.75	14.5	1.66	80.2	1.77
G9	75	106.2	0.40	16.1	0.24	90.1	0.43
G10	100	101.2	1.38	15.3	1.51	85.8	1.35
G11	100	100.6	1.01	15.3	0.90	85.3	1.03
G12	100	102.0	0.37	14.5	0.32	87.5	0.38

[a] All determinations in triplicate. USP doxepin hydrochloride Reference Standard Lot G-1 was used as a standard.

RSD of less than 20%. Compound IV decomposed to Compounds V and VI during injection. Compound IV is not likely to be present because it is an acid and the drug is a base, and it precedes the drug by 3–4 steps in the synthesis. None of the drug raw materials examined exhibited impurity peaks that exceeded the minimum quantifiable levels.

Organic Volatile Impurities

Some of the raw material samples were examined for organic volatile impurities. Several contained 100–200 ppm acetone, and one contained ethyl acetate. The chromatogram obtained from 1 of these samples and the corresponding IR spectrum are reproduced in Figure 4. None of the compounds limited by the USP (5) or proposed for limitation by the *European Pharmacopeia* (8) was detected in any of the samples.

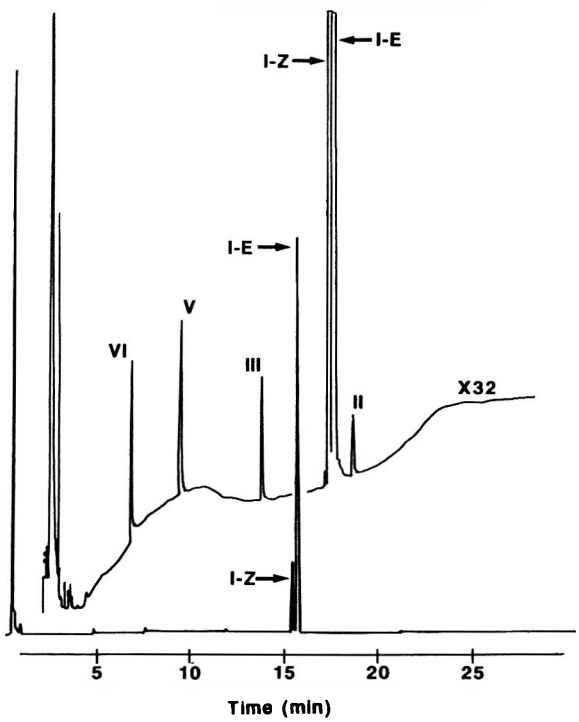

Figure 3. Gas chromatogram of (*E*)- and (*Z*)-doxepin (1 μg doxepin on-column) and 4 related compounds at levels of 0.5% by weight relative to the drug.

Conclusion

The selectivity and sensitivity of the GC methods described in this paper have been demonstrated. They are simpler than current pharmacopeial procedures and can be used with confidence for assay, isomer content, purity, and organic volatile impurities in doxepin hydrochloride and for assay and isomer content of capsules.

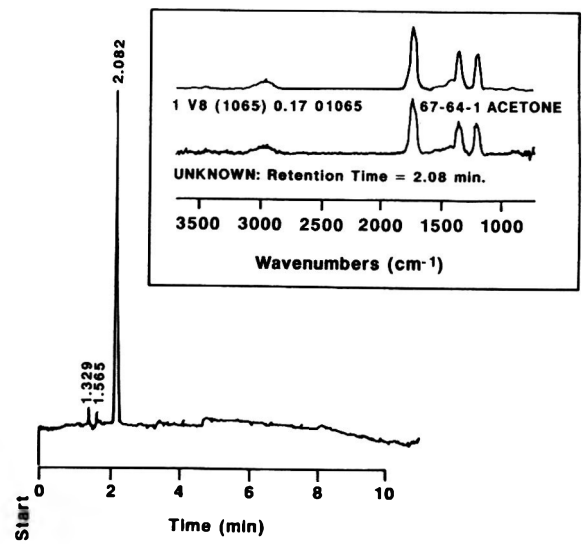

Figure 4. Chromatogram of Sample A1 showing ca 100 ppm acetone by the organic volatile impurities method. The inset compares the acetone IR spectra from the sample (bottom) and the FTIR library (top).

Acknowledgments

Solid-phase IR and NMR spectra were obtained by H. Beckstead and H. Avdovich, respectively.

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DRUGS

Liquid Chromatographic Determination of Sucralfate in Dental Cream

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A simple, rapid liquid chromatographic method is described for quantitative determination of sucralfate in dental cream. Analyses were performed on a weak anion exchange column with 0.6M ammonium sulfate–0.005M tetrabutylammonium hydrogen sulfate as the mobile phase and refractive index detection. The method was linear over a range of 0.0 to 20.0% sucralfate in dental cream. The coefficient of variation was 3.2%. Standard recoveries were concentration dependent and ranged from 97.2 to 104.9%.

Sucralfate, a basic aluminum salt of sucrose octasulfate, is marketed in the United States as Carafate, an orally-administered, gastrointestinal antiulcer drug. In addition, sucralfate suspensions may be effective in the treatment of mouth ulcers (1) and chemotherapy-induced mucositis (2).

The present paper describes a liquid chromatographic (LC) method that has been applied to dentifrices containing sucralfate that were investigated for potential antigingivitis activity.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Model 510 pump (Waters Chromatography, Div. of Millipore, Milford, MA 01757), equipped with SP6040 XR refractive index detector (Spectra-Physics, Santa Clara, CA 95051), Model 7120 injector valve with 100 µL sample loop (Rheodyne Inc., Cotati, CA 94928), and Shandon APS-Hypersil 2 column, 160 × 4.6 mm, 5 µm particle size (Keystone Scientific Inc., Bellefonte, PA 16823). Operating conditions: column temperature, ambient; mobile phase flow rate, 1.0 mL/min; detector range, ×64.

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Reagents

(a) *Chemicals and solvents.*—LC or analytical reagent grade. Tetrabutylammonium hydrogen sulfate (Aldrich Chemical Co., Milwaukee, WI 53233).

(b) *Mobile phase.*—0.6M Ammonium sulfate and 0.005M tetrabutylammonium hydrogen sulfate filtered through 0.2 µm filter, degassed under vacuum by sonication.

(c) *Standard.*—Sucralfate was obtained from Guilini Chemie (Germany) and used without special treatment.

(d) *Standard solution.*—Sucralfate (125 mg) was weighed into 25.0 mL volumetric flask and dispersed in 20 mL mobile phase. The pH was adjusted to 3 with concentrated sulfuric acid. The contents of the flask were diluted to volume with mobile phase and mixed well.

Sample Preparation

Approximately 1.25 g dental cream was accurately weighed, to the nearest milligram, into a 25.0 mL volumetric flask. Sample was introduced into the bottom of the flask without contamination of the walls of the neck as follows: The tip of a disposable Pasteur pipet was broken, leaving an opening wide enough to allow easy aspiration of the viscous dentifrice. The outside of the pipet was wiped clean and inserted through the neck into the bottom of the tared flask, where dentifrice was expelled.

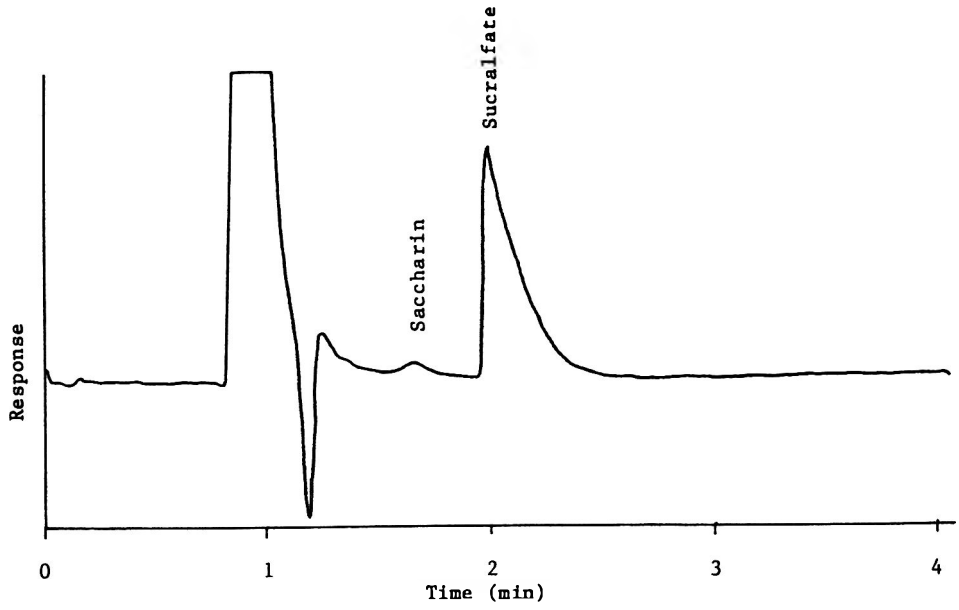

Figure 1. Liquid chromatogram of sucralfate (1.90 min) and saccharin (1.52 min).

The dental cream was dispersed in 20 mL mobile phase. The pH was adjusted to 3 with concentrated sulfuric acid. Contents of the flask were diluted to volume with mobile phase and mixed well. Sample was filtered through a 0.45 μm filter into a clean vial.

Samples for Forced Degradation Study

(a) *Acid hydrolysis.*—Sucralfate was refluxed 1 h in 0.1M sulfuric acid. Contents of the flask were quantitatively transferred to a volumetric flask with mobile phase. The pH was adjusted to 3 with concentrated ammonium hydroxide before dilution to volume with mobile phase.

Table 1. Analysis of recovery study samples

Spike level, %	Added, mg	Found, mg	Rec., %
0.0	0.00	0.00	—
	0.00	0.00	—
	0.00	0.00	—
2.5	34.47	36.33	105.4
	35.58	38.07	107.0
	32.35	33.06	102.2
5.0	67.35	68.76	102.1
	63.93	65.14	101.9
	63.30	62.16	98.2
10.0	126.8	126.4	99.7
	124.6	123.6	99.2
	125.6	124.1	98.8
20.0	250.6	247.3	97.2
	259.4	246.8	97.2

(b) *Base hydrolysis.*—Sucralfate was refluxed 1 h in 0.1M sodium hydroxide. Contents of the flask were quantitatively transferred to a volumetric flask with mobile phase. The pH

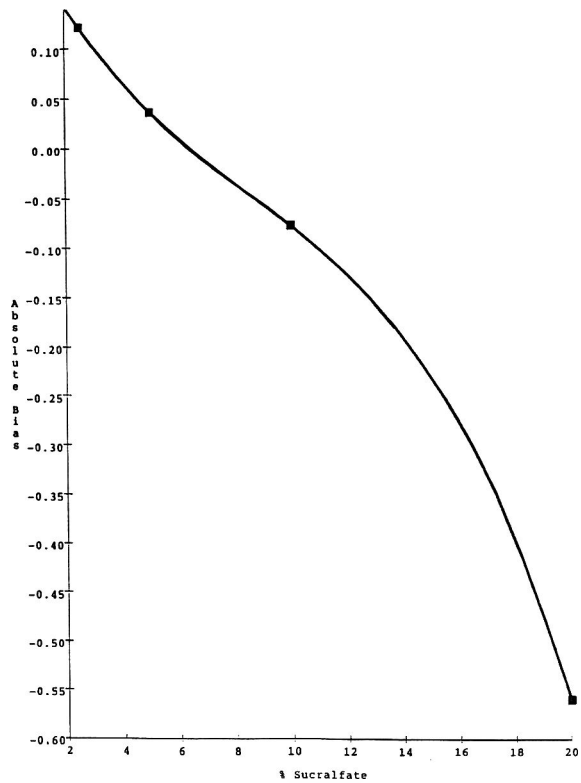

Figure 2. Plot of absolute bias vs percent sucralfate found in dental cream.

Table 2. Correction table for bias

Analytical result found, %	Correction
0.0–1.9	Subtract 0.2%
2.0–4.9	Subtract 0.1%
5.0–8.9	No correction
9.0–12.9	Add 0.1%
13.0–15.9	Add 0.2%
16.0–17.9	Add 0.3%
18.0–18.9	Add 0.4%
19.0–19.9	Add 0.5%
20.0	Add 0.6%

was adjusted to 3 with concentrated sulfuric acid before dilution to volume with mobile phase. The solution was filtered (0.45 μm) before injection.

(c) *Thermal degradation.*—Sucralfate was placed in a volumetric flask and stored 1 h at 180°C. Contents of the flask were dispersed in mobile phase. The pH was adjusted to 3 with concentrated sulfuric acid before dilution to volume with mobile phase. The solution was filtered (0.45 μm) before injection.

(d) *Chemical oxidation.*—Sucralfate was placed in a volumetric flask and treated 10 min with an acetic acid solution of peracetic acid. The pH was adjusted to 3 with concentrated sulfuric acid before dilution to volume with mobile phase.

Samples for Recovery Study

Placebo dental cream was spiked with sucralfate at levels corresponding to 0.0, 2.5, 5.0, 10.0, and 20.0%. Replicate analyses were performed as described above.

Determination

The LC column was equilibrated ca 1 h with mobile phase or until a stable baseline was achieved. Equal volumes of the standard and sample preparations were injected.

Calculation

The quantity of sucralfate was calculated by comparison of sample peak responses with standard peak responses as follows:

$$\% \text{ sulcralfate} = (Au \times Ws)/(10 \times As \times Wu)$$

where Au and As = peak area responses for the sample preparation and standard preparation, respectively; Ws = weight in milligrams of the sucralfate used in the standard; and Wu = weight in grams of dental cream.

Results and Discussion

A typical chromatogram is presented in Figure 1. Saccharin, a common ingredient in dental creams, eluted at 1.5 min ($k' = 0.8$) and was baseline separated from the sucralfate peak, which eluted at 2.0 min ($k' = 1.4$). The sucralfate capacity factor, although small, was a compromise between retention of the compound and minimization of the tailing factor.

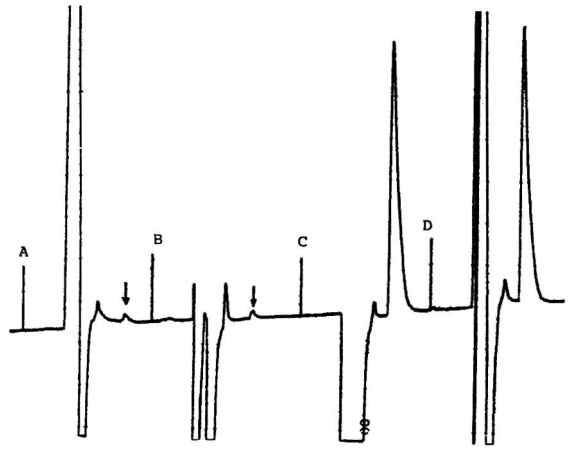

Figure 3. Chromatograms depicting the result of the forced degradation study: (A) thermal degradation, (B) acid hydrolysis, (C) base hydrolysis, and (D) chemical oxidation. The arrow indicates the sucralfate retention time.

Results of the recovery study are shown in Table 1. Because the sucralfate peak tailed slightly, the use of areas was recommended for quantitation. The method bias was concentration-dependent, ranging from +0.1% absolute (2.5% level) to –0.6% absolute (20.0% level). A plot of absolute bias vs percent sucralfate is presented in Figure 2. The asymptotic shape suggests that the dependence of bias on sucralfate level is solubility-related. This was consistent with known information on the compound and was supported by the observation of precipitation in the prepared 20.0% samples on standing. The appropriate correction factors for bias are presented in Table 2. The average coefficient of variation was 3.2%.

The calibration curve was linear over a range of 0.0–20.0% sucralfate in dental cream, with a correlation coefficient of 0.9999.

The forced degradation study was used to evaluate the method's ability to detect loss of parent compound when sucralfate decomposed (Figure 3). The acid hydrolysis and thermal degradation samples exhibited nearly complete loss of sucralfate. The base hydrolysis sample exhibited 4% loss, and the chemical oxidation sample exhibited no loss. The results were not surprising because sucrose, the backbone of sucralfate, is known both to decompose between 168 and 180°C and to undergo acid hydrolysis. Considering the rigorous conditions and low loss of parent compound in the base hydrolysis and chemical oxidation experiments, it seemed unlikely that these would be primary modes of degradation in dental cream stored under normal conditions.

In conclusion, this method provides an accurate and precise means of assay for sucralfate in dental cream with a minimum of sample preparation and analysis time.

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FEEDS

Liquid Chromatographic Determination of Vitamin D in Animal Feeds and Premixes

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A liquid chromatographic (LC) method has been developed for the determination of vitamin D (D_2 + D_3) in animal feeds and premixes. The sample is saponified with potassium hydroxide, and vitamin D is extracted with hexane and isomerized to isotachysterol with 10M HCl in 2-butanol. LC determination of isotachysterol to quantitate vitamin D is carried out on a reversed-phase column with acetonitrile–methanol (90 + 10) as the mobile phase and ultraviolet detection at 301 nm. The detection limit of the method is 1 IU/g. This method can also be used for the determination of vitamin D_2 and D_3 separately.

Animal feeds and premixes are fortified with vitamin D, which is important for the growth and development of animals. A number of techniques, including thin-layer chromatography (1), gas-liquid chromatography (2, 3), bioassay (4, 5), and spectrophotometry (6, 7), have been described for the determination of vitamin D in feedstuffs and vitamin premixes. Liquid chromatography (LC) has become the method of choice for the determination of vitamin D in different matrixes. All the LC methods reported for the analysis of vitamin D in feeds and premixes require saponification and extraction, sample cleanup, and finally, quantitative determination by LC (8–15).

Premixes generally contain high levels of vitamin D and do not require extensive cleanup. Animal feeds, however, have low levels of vitamin D, and require extensive cleanup to remove interfering substances before any quantitative determination of vitamin D can be carried out by LC. Ray et al. (8) described an LC method that does not require any cleanup, but their method is applicable only to premixes. Lein et al. (9) also described an LC method that requires only a simple silica Sep-Pak cleanup before LC analysis but is applicable only for premixes. Other LC methods reported for the analysis of animal feeds and premixes require extensive cleanup before LC quantitation (10–14). The present official AOAC method also requires extensive cleanup after saponification and extraction. Initial cleanup on an alumina column is followed by additional cleanup on reversed-phase LC (15).

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Because of the similar biological activities of vitamin D_2 and D_3, little emphasis has been given to the separation of the 2 vitamins in the analysis of feeds and premixes. For chicken feeds, however, it is important to distinguish between the 2 vitamins because only vitamin D_3 is active for this species (14). Kobayashi et al. (14) reported a method that allows the separation of vitamin D_2 and D_3 but requires an additional LC step. The only other method reported for successful separation of vitamin D_2 and D_3 requires 4 cleanup steps before LC (13).

LC methods for the determination of vitamin D in milk and infant formula have previously been reported from our laboratory. In these methods, vitamin D is isomerized to isotachysterol after saponification and extraction and then determined by LC (16, 17). A similar approach has now been applied for the determination of vitamin D in feeds and premixes.

METHOD

Reagents and Standards

(a) *Chemicals.*—LC grade methanol and acetonitrile, analytical grade chloroform, reagent grade potassium hydroxide (Fisher Scientific Co., Springfield, NJ 07081). Vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) (Sigma Chemical Co., St. Louis, MO 63178).

(b) *Aqueous KOH solution.*—Dissolve 400.0 g reagent grade KOH in water, cool, and dilute to 500 mL with water.

(c) *Alcoholic KOH solution.*—Dissolve 15.0 g reagent grade KOH in 400 mL water and dilute to 500 mL with 95% ethanol.

(d) *Ethanolic pyrogallol solution.*—Dissolve 1.0 g pyrogallol in 100 mL 95% ethanol.

(e) *Isomerization reagent.*—Prepare ca 10M HCl in 2-butanol by bubbling dry HCl gas in 2-butanol cooled to 0°C in hood. Store in freezer below 0°C.

(f) *Aqueous sodium carbonate solution.*—Dissolve sodium carbonate in distilled water at room temperature to prepare saturated solution.

(g) *Vitamin D standard solution.*—(*1*) *Stock solutions*: Dissolve separate 100 mg portions of vitamin D_2 and D_3 in methanol and dilute each to 200 mL with methanol. (*2*) *Intermediate solutions*: Dilute 5 mL of each stock solution separately to 100 mL with methanol. (*3*) *Working standard solutions*: Dilute separate 1 mL portions of each intermediate standard to 50 mL

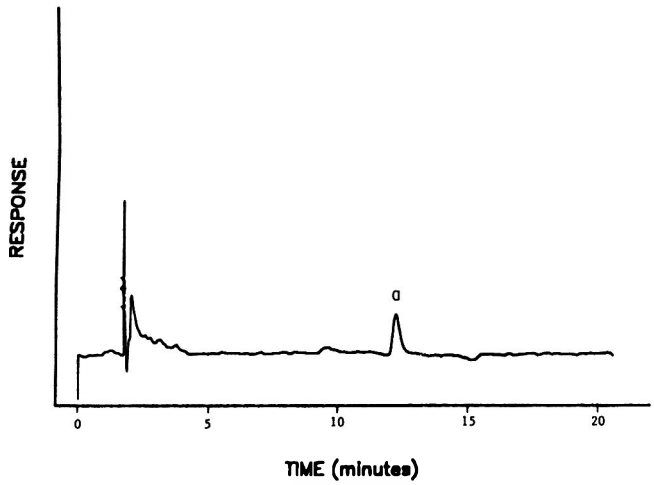

Figure 1. Chromatogram of standard isotachysterol D_3 (a).

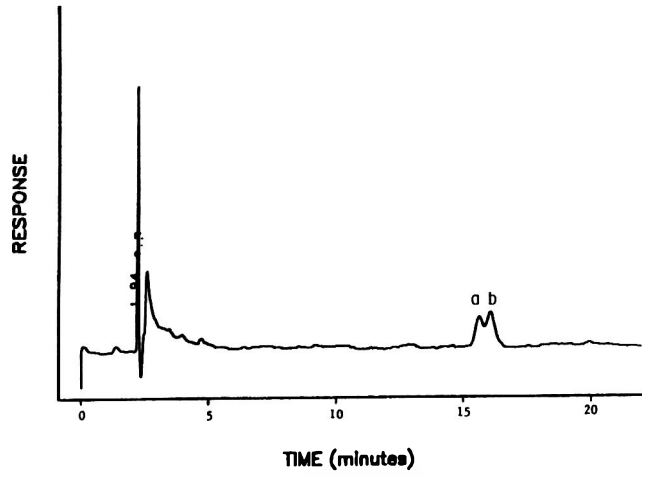

Figure 2. Chromatogram of standard isotachysterol D_2 (a) and D_3 (b).

with methanol to provide concentration of 20 IU/mL (0.5 μg/mL).

Apparatus

(a) *Centrifuge tubes.*—25 mL conical centrifuge tubes with glass stoppers were used for isomerization.

(b) *Vortex mixer.*—Bronwill vortex mixer, or equivalent.

(c) *LC system for quantitation.*—Laboratory Data Control Constametric III pump equipped with Model 7105 syringe injection valve with 200 μL sample loop (Rheodyne, Inc., Cotati, CA 94931), SpectroMonitor III variable-wavelength detector (LDC, Riviera Beach, FL 33404), and Model 3390A integrator (Hewlett-Packard, Palo Alto, CA 94304). Monitor eluant at 288 and 301 nm.

(d) *Chromatographic columns.*—Supelcosil LC-18-DB, 5 μm, 25 cm × 4.6 mm id, and guard column LC-18-DB, 5 μm sphere, 2 cm × 4.6 mm (Supelco, Inc., Bellefonte, PA 16823).

Saponification and Extraction

Saponify feed sample or premix and extract as described earlier (16). Place 1–2 g feed or 0.5 g premix in 250 mL low-actinic-glass Erlenmeyer flask. Add 50 mL ethanolic pyrogallol solution and 15 mL aqueous KOH solution. Place small magnetic stirring bar in flask and saponify mixture overnight at room temperature with slow constant stirring. Decant saponified mixture to 500 mL separatory funnel. Add 50 mL hexane to flask, stir well, and let settle for 5 min. Decant hexane to same separatory funnel. Shake funnel vigorously to extract vitamins into hexane layer. Remove hexane layer and extract aqueous layer again with 50 mL hexane. Combine hexane extracts and wash twice with 60 mL alcoholic KOH solution. Wash combined hexane extracts with 50 mL portions of water until there is no color to phenolphthalein in aqueous layer. Remove water from hexane extract with anhydrous sodium sulfate. Concentrate extract to ca 5 mL on rotary evaporator while maintaining bath temperature at ca 40°C. Transfer concentrated extract to 40 mL centrifuge tube and evaporate to dryness under slow stream of nitrogen. Redissolve extract in 20 μL methanol.

Isomerization

Cool standard vitamin D or extract dissolved in 200 μL methanol to 5°C. Add 0.6 mL derivatizing reagent to centrifuge tube containing extract or standard, shake well on vortex mixer for 15–20 s, and leave at 5°C for 3 min. Shake sample tube on vortex mixer every 30 s for 3 min. Add 5 mL aqueous saturated sodium carbonate solution to tube and shake well on vortex mixer to destroy any excess reagent.

Add 2 mL chloroform to tube containing derivatized sample and shake well on vortex mixer. Pass chloroform layer through small column filled with anhydrous sodium sulfate (use disposable pipet for this purpose). Extract aqueous layer again with 2 mL chloroform and pass chloroform extract through same column. Collect all eluants in 40 mL centrifuge tube, evaporate to dryness under slow stream of nitrogen at room temperature, and then redissolve in 500 μL methanol.

Quantitative LC Analysis

The LC system was set up as detailed in *Apparatus* section. The following operating conditions were used: ambient temperature; mobile phase, acetonitrile–methanol (90 + 10); flow rate, 1.8 mL/min; detector, 301 nm; sensitivity, 0.05 AUFS. Isomerized vitamin D (isotachysterol) was injected into LC to determine its actual retention time (12.0 ± 0.5 min); 10 μL sample extract was injected into LC and isotachysterol was quantitated by peak height. All peaks eluted in 25 min and next injection could be made.

Results and Discussion

Vitamin D in premixes and feeds was quantitated by determination of isotachysterol formed by isomerization of vitamin D. A similar approach was previously applied to the determination of vitamin D in milk and infant formula (16, 17). Isotachysterol has absorption maxima at 278, 288, and 301 nm.

Table 1. Determination of vitamin D_3 in premixes and animal feeds

No.	Sample	Guarantee, IU/g	Found, IU/g[a]	Vitamin D_3 added, IU/g	Rec., %
1	Premix	1328.6	301.3 ± 15.5	1000	92.0 ± 11.4
2	Premix	1455.3	1388.0 ± 83.0	—	—
3	Feed	13.2	11.7 ± 0.7	10	87.0 ± 4.6
4	Feed	11.0	11.8 ± 1.1	—	—

[a] Av. ± standard deviation, n = 3.

Although the UV absorption of isotachysterol is strongest at 288 nm, detection in earlier reports was done at 301 nm because of fewer interferences at this wavelength. The ratio of isotachysterol peaks at 288 and 301 nm is 1.3. The detection of isotachysterol at both wavelengths can serve as a useful indication of purity of the isotachysterol peak.

A linear calibration curve for isotachysterol D_3 (isotachysterol formed by the isomerization of vitamin D_3) was obtained by plotting the mean peak heights vs isotachysterol concentration in the 5–50 IU range. The correlation coefficient was 0.998.

The saponification step is similar to that used for milk fortified with vitamin D (16). Vitamin D is isomerized to isotachysterol as for infant formulas (17). The final step, i.e., quantitation, is performed by reversed-phase LC with UV detection at 301 and 288 nm. Figure 1 shows the chromatogram of standard isotachysterol D_3. Vitamin D_2 was also isomerized in a similar manner.

Under these LC conditions, isotachysterol D_2 and D_3 were not very well separated. By changing the flow rate of the mobile phase from 1.8 to 1.2 mL/min, it was possible to separate isotachysterol D_2 and D_3. Figure 2 shows a chromatogram of standard isotachysterol D_2 and D_3 at the lower flow rate of 1.2 mL/min. Because the aim of this work was to determine quantitatively the total vitamin D ($D_2 + D_3$) in premix and feed, the LC system was operated at a flow rate of 1.8 mL/min, which speeds up the determination. When it is necessary to identify and quantitate vitamin D_2 and D_3 separately, a slower flow rate can be used.

A number of premix and feed samples were analyzed by this procedure. Figure 3 shows a chromatogram of a premix, monitored at 301 nm, with a label claim of 1328.6 IU of vitamin D_3/g. LC eluant was also monitored by UV to detect isotachysterol at 288 nm, and the ratio of the isotachysterol peaks at 288 and 301 nm was found to be 1.3, suggesting that no interfering peaks were coeluting. Table 1 shows the results of vitamin D analysis of 2 commercial premix samples. Sample 1 contained only 301 IU/g, although the label claim was 1328.6 IU/g. Sample 2 contained 1388 IU/g, although the label claim was 1455.3 IU/g. To determine the accuracy of the method, Sample 1 was spiked with 1000 IU/g of standard vitamin D_3 to provide a final concentration of 1301 IU/g, and vitamin D was determined. The recovery of spiked sample was 92.0% with a standard deviation of 11.4%.

Two commercial feeds were also analyzed similarly (Table 1). Samples 3 and 4 contained 11.7 IU/g (label claim, 13.2 IU/g) and 11.8 IU/g (label claim, 11.0 IU/g), respectively. Figure 4 shows a chromatogram of a feed sample, monitored at 301 nm, that had a label claim of 13.2 IU vitamin D_3/g. The LC eluant was again monitored at 288 nm to confirm the absence of any interfering peak coeluting with the isotachysterol peak. Feed Sample 3 was then spiked with vitamin D_3 to provide a total concentration of 21.7 IU vitamin D/g, and vitamin D_3 was determined. The recovery of the spike sample was 87% with a standard deviation of 4.6%.

In all 4 samples of premix and feeds analyzed, no interfering peaks were observed, and, therefore, no cleanup step was re-

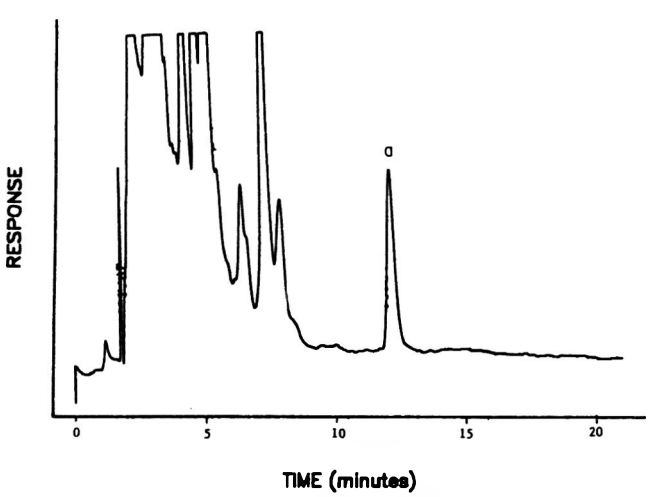

Figure 3. Chromatogram of a premix sample. Peak (a) is isotachysterol D.

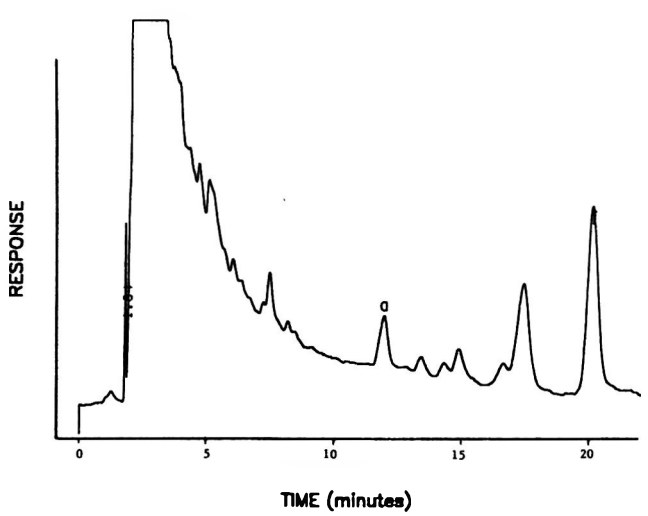

Figure 4. Chromatogram of an animal feed sample. Peak (a) is isotachysterol D.

quired. On the basis of the reports in the literature, however, the presence of interfering peaks in other commercial samples of feeds and premixes cannot be ruled out completely. In such cases, the isotachysterol D fraction can be collected and analyzed by normal-phase LC as for infant formula (17).

In conclusion, this method represents a simple and reliable approach for the quantitative analysis of vitamin D in animal feeds and premixes. Saponification of the sample at room temperature avoids the problem of conversion of vitamin D to previtamin D. The isomerization of vitamin D to isotachysterol helps to minimize any loss due to the unstable nature of vitamin D and also increases the sensitivity and selectivity of the method.

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FEEDS

Liquid Chromatographic Determination of Unbound and Acetone-Soluble Bound Gossypol in Cottonseed Meals and Mixed Feeds

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A liquid chromatographic method has been developed for the determination of unbound and acetone-soluble bound gossypol in cottonseed meals and mixed feeds at levels of 0.5 ppm. The method involves extraction with aqueous acetone in the presence of ascorbic acid, hydrolysis of the "soluble bound" forms with hydrochloric acid, partitioning into chloroform, and chromatographic separation on a 10 μm C_{18} column by step gradient elution using a methanol–water mobile phase acidified with phosphoric acid. With minor modifications, the method permitted discriminate determination of unbound gossypol and acetone-soluble hydrophilic and lipophilic forms of bound gossypol. The gossypol peak was characterized by on-line spectral scanning and absorbance rationing. Overall relative standard deviation was 6.7% and overall recovery was 98.1 ± 3.3%. When the method was applied to several cottonseed meal samples, results were inconsistent with those obtained by the official American Oil Chemists' Society method for "free" gossypol determination.

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Gossypol, 1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl(2,2′-binaphthalene)-8,8′-dicarboxaldehyde, is a toxic compound that constitutes approximately 0.4–1.7% by weight of glanded cottonseed, depending on both variety and environmental parameters (1). Processing of cottonseed, however, results in considerable de-

activation of gossypol because of numerous interactions and molecular transformations that the compound undergoes through the action of heat, moisture, air oxygen, compression, and friction (2).

During processing, most gossypol reacts, through its aldehyde groups, with the free amino groups of proteins. The resulting condensation products, collectively termed "bound" gossypol, are not toxic and represent the aqueous acetone-insoluble portion of gossypol present in cottonseed meals. Gossypol also reacts with other seed constituents (amino acids, peptides, phospholipids, etc.) to form the corresponding condensation products that are soluble in aqueous acetone and represent the so-called "soluble bound" gossypol, which is toxic (3). Moreover, some gossypol is oxidized and/or degraded to various products; most products have not been identified and differ in their properties and spectral characteristics (2). The remaining unbound gossypol and those gossypol-related compounds soluble in aqueous acetone that have an aldehyde moiety represent the conventionally termed "free" gossypol (4).

Free gossypol is solely responsible for the toxicity to monogastric animals (5–8) and young ruminants (9–11) of cottonseed meal, a low-cost and high-protein material. Therefore, its accurate and precise determination is important both for process control by the industry and for feed inspection.

Various methods for the determination of free gossypol in cottonseed meals have been published. Early methods included gravimetric (12, 13), titrimetric (14), polarographic (15), and chemiluminescent (16) procedures. Current methods are spectrophotometric procedures based on the reaction of gossypol with antimony trichloride (17, 18), *p*-anisidine (19), phloroglucinol (20, 21), or aniline (4, 22–24). Methods for mixed feeds have also been described (20, 25), but these are actually slight modifications of spectrophotometric procedures originally developed for cottonseed materials.

Most toxicity studies with gossypol have been based on spectrophotometric procedures, primarily those proposed by the American Oil Chemists' Society (AOCS) (4). However, in view of the toxicity of gossypol to animals and the levels normally encountered in cottonseed meals and mixed feeds, the suitability of these methods remains in doubt. Some findings suggest that the free gossypol determined by such methods is not always a true measure of cottonseed meal toxicity, because the various compounds comprising free gossypol differ in physiological activity (26–29). Moreover, false positives are frequently obtained for materials known to be free of gossypol (19, 30–32). Further, the sensitivity of these methods can hardly satisfy the requirements for mixed feed analysis.

The literature contains highly sensitive and specific methods for gossypol analysis, based on nuclear magnetic resonance (33, 34), gas-liquid chromatography (35, 36), and liquid chromatography (LC) (37–45). These methods, however, cannot be used for free gossypol determination because other physiologically active gossypol-related compounds in cottonseed meal are not measured concurrently.

Recently, new methods capable of determining free gossypol in cottonseed meal by second-derivative spectrophotometry (46) and LC (47) have been published. The former is based on hydrolysis of the soluble bound gossypol with hydrochloric acid, partitioning into chloroform, and second-derivative spectrophotometry; the latter involves hydrolysis with acetic acid, reaction with 3-amino-1-propanol, and chromatography of the resulting complexes eluting as a single peak. Both methods, although lacking the sensitivity required to monitor the low levels of free gossypol encountered in mixed feeds, are useful as alternatives to the AOCS method for cottonseed meal analysis. However, none of them can differentiate the individual compounds comprising free gossypol.

This study deals with the development of an LC method for determining unbound and acetone-soluble hydrophilic and lipophilic forms of bound gossypol in cottonseed meals and mixed feeds. Although other transformation and/or degradation products of gossypol formed during cottonseed processing cannot be simultaneously determined, the method offers the opportunity to evaluate the physiological activity of these discriminated gossypol classes and, therefore, provides better access to the problem of cottonseed meal toxicity.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Perkin-Elmer Series 3 modular chromatograph equipped with 2 reciprocating pumps controlled by microcomputer, power solvent mixer, LC-100 column oven, LC-55-B single-beam variable-wavelength UV-vis spectrophotometer, and 023 variable-span recorder. Perkin-Elmer LC-55-S digital scanner for monitoring corrected spectra of the eluted compounds on stop-flow conditions; appropriate peak captured in detector cell by shutting off pump power and simultaneously closing valve located in front of Rheodyne 7105 injector.

(b) *LC slurry packer.*—Magnus Scientific (United Kingdom), Model P6060.

(c) *Chromatographic column.*—Stainless steel, 25 × 0.46 cm, hand-packed with 10 μm Spherisorb ODS2 (Phase Sep, United States) at 6000 psi by downward slurry packing technique (48). Suspending medium: methanol–water (80 + 20, v/v) containing 0.0002 g sodium acetate/mL (Merck, Germany).

(d) *Laboratory mill.*—Retsch KG (Germany), Model SK1, equipped with 1 mm screen.

(e) *Water bath.*—Tamson (The Netherlands), Model T.X.V. 45, constant temperature control (accuracy, ± 0.1°C).

(f) *Evaporator.*—Buchi (Switzerland), Model Rotavapor-R.

(g) *Vortex mixer.*—Heidolf (Germany), Model REAX 1 R.

Reagents

(a) *Solvents.*—LC-grade methanol (Burdick & Jackson, United States) and analytical grade acetone, chloroform, acetonitrile, hydrochloric acid, phosphoric acid, and petroleum ether, 40–60°C (Merck).

(b) *Aqueous acetone–ascorbic acid solution.*—Dissolve 2.5 g ascorbic acid (Merck) in 150 mL water, and mix with 350 mL acetone.

(c) *Ascorbic acid solution.*—Dissolve 1.5 g ascorbic acid in 500 mL water.

(d) *Acetonitrile-extracted petroleum ether.*—Shake 100 mL petroleum ether with two 50 mL portions of acetonitrile, and keep top layer.

(e) *Gossypol standard solution.*—Weigh ca 12 mg gossypol acetic acid (89.62% gossypol; Makor Chemicals, Israel), dissolve, and dilute to 50 mL with acetonitrile (stock solution). Store at –25°C and prepare fresh weekly. Each work day, dilute aliquots of this solution to prepare working solutions in 0.3–8 μg/mL range.

(f) *Mobile phase.*—Purify glass-distilled water by passing it through C_{18} column. Separately acidify appropriate volumes of LC-grade methanol and purified water, using 0.1 mL phosphoric acid for each 100 mL solvent. Fill pump reservoirs with acidified solvents, and degas thoroughly by applying magnetic stirring under vacuum.

Sample Preparation

Grind ca 50 g cottonseed meal or mixed feed in laboratory mill through 1 mm screen; avoid overheating.

Determination

Accurately weigh ca 2 g sample material into 250 mL glass-stoppered Erlenmeyer flask, and cover bottom of flask with glass beads. Add 100 mL aqueous acetone–ascorbic acid solution, stopper flask, and shake vigorously on wrist-action mechanical shaker for 1 h. Filter through Whatman No. 40 filter paper, or equivalent, and discard first 5 mL filtrate. Use separate 25 mL aliquots of filtered extract for determination of unbound and acetone-soluble bound gossypol, unbound gossypol, and unbound and acetone-soluble lipophilic form of bound gossypol.

(a) *Unbound and acetone-soluble bound gossypol.*—Pipet 25 mL extract into 50 mL volumetric flask, and add 0.05 mL hydrochloric acid. Place flask in 65°C water bath, allow to equilibrate, stopper flask, and heat 1 h. Remove flask from bath, cool to room temperature, and transfer contents into 250 mL separatory funnel. Add 50 mL chloroform, 100 mL ascorbic acid solution, and 1 mL hydrochloric acid to funnel. Shake funnel for 3 min to partition, let stand for 5 min, and filter lower organic layer through anhydrous sodium sulfate on Whatman No. 40 paper into 100 mL glass-stoppered flask. Rinse sodium sulfate by filtering 5 mL chloroform through paper, and evaporate combined filtrates to dryness by rotary evaporation at 30°C. Remove traces of chloroform by nitrogen stream, and dissolve remaining residue in acetonitrile. Volume depends on expected unbound and acetone-soluble bound gossypol content: for samples with <2 ppm, use 0.5 mL; for 2–10 ppm, 1 mL; for 10–40 ppm, 4 mL; for 40–100 ppm, 10 mL; for 100–400 ppm, 40 mL; and for >400 ppm, 100 mL.

(b) *Unbound gossypol.*—Pipet 25 mL extract into 250 mL separatory funnel and proceed as in (a), omitting heating with hydrochloric acid.

(c) *Unbound and acetone-soluble lipophilic form of bound gossypol.*—Pipet 25 mL extract into 250 mL separatory funnel and proceed as in (b), omitting residue reconstitution into acetonitrile. Dissolve residue in 25 mL aqueous acetone–ascorbic acid solution and proceed as in (a).

(d) *Optional cleanup for samples with <10 ppm gossypol.*—Transfer acetonitrile extract into 15 mL tube by using two 1 mL portions of acetonitrile to rinse flask walls; add 3 mL acetonitrile-extracted petroleum ether, mix by vortexing at high speed for 30 s, and centrifuge 1 min at 1000 × *g*. Discard top layer, and evaporate bottom layer to dryness by using gentle nitrogen stream. Redissolve remaining residue in 0.5 or 1 mL acetonitrile.

(e) *Liquid chromatography.*—Set spectrophotometer at 254 nm, recorder at 0.050 AUFS, and flow rate at 1.5 mL/min. Maintain oven at 30°C to isolate column from fluctuations in ambient temperature. Program mobile phase composition in relation to time as follows: 2 min concave gradient from 80:20 (acidified methanol–water, v/v) to 84:16, 2 min convex gradient to 92:8, 6 min isocratic at 94:6, and 8 min equilibration at initial conditions. During concave and convex gradients, mobile phase composition changed as function of second power and second root, respectively, of quotient "elapsed time/time segment."

Inject 25 μL of both sample and working standard solutions. Under described conditions, gossypol elutes at 8 min. After each day's work, flush column with purified water until eluate is free of acidity. Keep column filled with methanol–water (80 + 20, v/v) when not in use.

Calculation

Measure peak heights; plot vs concentration of injected working solutions; and record slope, intercept, and least squares fit of standard curve. Use standard curve slope and intercept data to compute concentration of gossypol in sample extracts from recorded peak heights. Calculate unbound and acetone-soluble bound gossypol, unbound gossypol, and unbound and acetone-soluble lipophilic form of bound gossypol content of sample by following formula:

$$\text{Gossypol content (ppm)} = (C \times V \times 4)/W$$

where C = concentration (μg/mL) of gossypol in final sample extract; V = volume (mL) of acetonitrile used for dilution; and W = weight (g) of sample analyzed.

Calculate acetone-soluble lipophilic form of bound gossypol by subtracting unbound gossypol from unbound and acetone-soluble lipophilic form of bound gossypol. Calculate acetone-soluble hydrophilic form of bound gossypol by subtracting unbound and acetone-soluble lipophilic form of bound gossypol from unbound and acetone-soluble bound gossypol. Prepare new standard curve each working day.

Results and Discussion

The extraction of unbound and acetone-soluble bound gossypol from cottonseed meal and mixed feed samples, the hy-

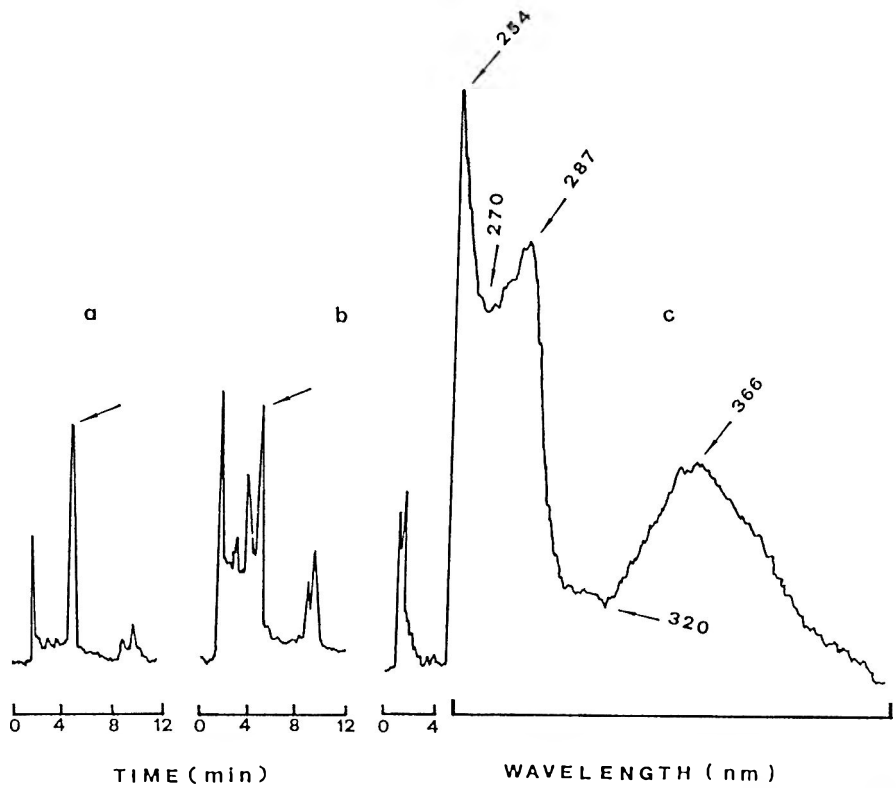

Figure 1. Typical chromatograms of cottonseed meal samples run isocratically: (a) cottonseed meal containing 321 ppm gossypol, (b) cottonseed meal containing 37 ppm gossypol, and (c) absorbance scan of peak apex. LC conditions: mobile phase, 0.1% phosphoric acid in methanol–water (88 + 12, v/v); column, 25 × 0.46 cm, C_{18}, 10 μm; temperature, 30°C; flow rate, 1.5 mL/min; wavelength, 254 nm; recorder sensitivity, 0.050 AUFS; chart speed, 0.25 cm/min; and injection volume, 25 μL.

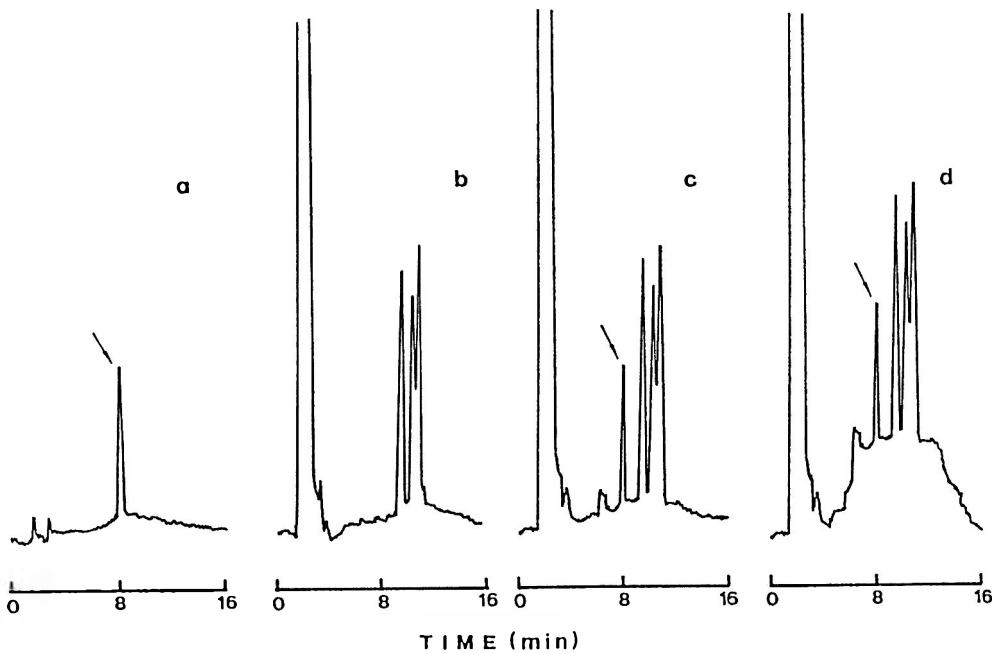

Figure 2. Typical chromatograms of standard gossypol solution and mixed feed samples run by step gradient programming: (a) standard gossypol solution, 2 μg/mL; (b) blank mixed feed; (c) blank mixed feed spiked with gossypol at 2 ppm level; (d) as in (c) but run with unpurified mobile phase water. LC conditions: step gradient using methanol and water, each containing 0.1% phosphoric acid; column, 25 × 0.46 cm, C_{18}, 10 μm; temperature, 30°C; flow rate, 1.5 mL/min; wavelength, 254 nm; recorder sensitivity, 0.050 AUFS; chart speed, 0.25 cm/min; injection volume, 25 μL.

Table 1. Absorbance wavelength ratios of gossypol peak

Point	Absorbance ratio			
	254/320	270/320	287/320	366/320
Peak apex	7.7	4.8	5.7	2.8
Peak leading	7.5	4.6	5.6	2.8
Peak tail	7.8	4.7	5.7	2.9

drolysis of the soluble bound forms, and the liquid-liquid partition of the liberated gossypol were all carried out in the presence of ascorbic acid, a minor modification of a recently published analytical scheme (46) for gossypol analysis. Without ascorbic acid, the percent recovery of gossypol was comparatively low, varying from 58 to 78%, whereas a 72–95% variation was noted in analyzing aqueous solutions of standard gossypol. Considering the strong antioxidant properties of gossypol (49, 50) and taking into account stability studies in various solvents (38, 51), it might be assumed that the almost quantitative recoveries found after ascorbic acid addition were solely due to its protective action against both oxygen content of the solvents and any oxidizing constituent of feed samples. It is known, however, that 2 of the 6 hydroxyl groups of gossypol have distinct acidic properties leading to formation of metal salts (2). Therefore, the protective action of ascorbic acid might be attributed not only to its reducing power but also to its competitive tendency to form stable metal salts (52).

LC of cottonseed meal extracts was initially attempted under isocratic conditions using a mobile phase of methanol–water (88 + 12, v/v) containing 0.1% phosphoric acid (Figure 1a). Under these conditions, however, baseline separation could not be effected unless samples contained more than 80 ppm gossypol (Figure 1b). Because a mobile phase change to 87% methanol dramatically influenced the retention behavior of gossypol by preventing its elution from the column, a solvent gradient was finally applied.

Table 2. Recovery data for LC analysis of gossypol in mixed feeds

Spiking level	Gossypol added, ppm[a]	Mean gossypol found, ppm (±SD)
0	0	11.6 (±0.23)
1	14.5	25.1 (±1.34)
2	28.9	41.8 (±0.65)
3	43.4	54.6 (±0.94)
4	57.8	67.8 (±0.17)

[a] Three 2 g replicates.

Gossypol could be adequately resolved by solvent programming; the recorded peaks were sharp, and analysis time was not excessive (Figures 2a, 2b, and 2c). However, the mobile phase water in this case had to be further purified through a C_{18} column; otherwise, a baseline rise appeared midway through each run even when no sample had been injected (Figure 2d). This rise was found to be due to UV-absorbing impurities of the distilled water that concentrated on the top of the column during equilibration time and eluted once a particular concentration of methanol was reached.

Peak identity in cottonseed meal samples was tested by capturing the leading edge (Figure 1c), apex, and tail of the peak in the detector cell and applying absorbance ratio technique. Because of inherent limitations of the existing equipment, the absorbance ratio technique was applied under isocratic conditions, but absorbance was measured at 5 wavelengths to allow setting up 4 ratios for more positive corroboration; close resemblance of the absorbance ratios at each capturing indicated peak integrity (Table 1).

Regression analysis of the data obtained by running a series of working solutions showed the response to be linear within the range studied (0.050 AUFS, 25 µL injection, $y = 0.019 + 13.077x$, correlation coefficient = 0.9993, where y = peak height [mm] and x = gossypol concentration [µg/mL]). The

Table 3. Precision data for LC analysis of gossypol in mixed feeds

Day	Gossypol found, ppm			Mean value, ppm	SD	RSD, %
1	15.0	14.0	13.4			
	14.5	13.8	14.0	14.1	0.6	5.3
2	14.8	14.2	14.6			
	14.6	15.2	14.8	14.7	0.3	3.9
3	14.1	15.8	15.6			
	15.7	16.2	16.8	15.7	0.9	6.0
Overall				14.8	0.9	6.4

Variance estimates:	Source	RSD, %
	Within day	4.3
	Between days	5.1
	Overall	6.7

Table 4. Unbound and acetone-soluble lipophilic and hydrophilic forms of bound gossypol in cottonseed meals as determined by the proposed LC method[a]

Sample	Gossypol, ppm			"Soluble-bound" gossypol, ppm	
	Unbound & soluble-bound	Unbound	Unbound & lipophilic bound	Lipophilic form	Hydrophilic form
Solvent-extracted meal, 1990 crop	373.0	138.5 (37.1%)	296.3	157.8 (42.3%)	76.7 (20.6%)
	321.5	103.0 (32.0%)	233.7	130.7 (40.6%)	87.8 (27.3%)
	354.0	109.5 (30.9%)	282.5	173.0 (48.9%)	71.5 (20.2%)
Solvent-extracted meal, 1989 crop	238.7	45.1 (18.9%)	175.4	130.3 (54.6%)	63.3 (26.5%)
	238.7	58.4 (24.5%)	172.1	113.7 (47.6%)	66.6 (27.9%)
	293.0	48.1 (16.4%)	161.5	113.4 (38.7%)	131.5 (44.9%)
Screw-pressed meal, 1989 crop (single-pressing)	568.6	51.2 (9.0%)	319.7	268.5 (47.2%)	248.9 (43.8%)
Screw-pressed meal, 1989 crop (double-pressing)	359.6	33.8 (9.4%)	241.4	207.6 (57.7%)	118.2 (32.9%)
	100.4	15.4 (15.3%)	65.6	50.2 (50.0%)	34.8 (34.7%)

[a] Values in parentheses are percentages of unbound and "soluble-bound" gossypol. All values are averages of 2 determinations.

high extinction of gossypol at 254 nm permitted quantitations down to 0.5 ppm, but awkward chromatograms were frequently recorded at such low concentrations because of the presence of very high neighboring to gossypol peaks. "Clean" chromatograms (Figures 2b and 2c) could be recorded by submitting the acetonitrile extract, before injection, to partitioning with petroleum ether that had been pre-extracted with acetonitrile; this pre-extraction removed petroleum ether impurities having retention behavior similar to that of the undesirable peaks.

In studying the accuracy of the analytical process, a recovery experiment was also evaluated. In this experiment, 12 of 15 samples from feed consisting of soybean meal, corn meal, cottonseed meal, dicalcium phosphate, sodium chloride, and vitamin–mineral premix were spiked with gossypol at 4 levels, using appropriate quantities from a gossypol–acetic acid stock solution. Least-squares and regression analysis of the data obtained by analyzing the spiked samples (Table 2) showed that the relationship between "added" and "found" was adequately described by a linear regression (correlation coefficient = 0.9969). The intercept (12.0) of the regression line ($y = 12 + 0.976x$) actually is the value in ppm predicted for the unspiked sample without recovery correction. Because it was found by the t test to be not significantly different from the arithmetic mean (11.6 ppm) of the values obtained by analyzing the unspiked samples, recovery evaluation was based on the data from both the spiked and unspiked samples (Table 2).

Least-squares and regression analysis of these data showed that linearity was quite acceptable (correlation coefficient = 0.9984); consequently, the slope (0.982 ± 0.033) of the regres-

Table 5. "Soluble-bound" gossypol in cottonseed meals determined by derivative and proposed LC method[a]

Sample	"Soluble-bound" gossypol, ppm	
	Derivative method	LC method
Solvent-extracted meal, 1990 crop	204	234.5
	211	218.5
	220	244.5
Solvent-extracted meal, 1989 crop	170	193.6
	171	198.3
	227	244.9
Screw-pressed meal, 1989 crop (single-pressing)	488	517.4
Screw-pressed meal, 1989 crop (double-pressing)	302	325.8
	75	85.0

[a] All values are averages of two 2 g replicates.

Table 6. Gossypol content of cottonseed meals as determined by AOCS method, derivative method, existing LC method, and proposed LC method[a]

Sample	"Free" gossypol, ppm			
	AOCS method	Derivative method	Existing LC method	Unbound & soluble-bound gossypol, ppm, by proposed LC (LC/AOCS × 100)
Solvent-extracted meal, 1990 crop	520	531	378	373.0 (73%)
	557	520	318	321.5 (58%)
	591	578	328	354.0 (55%)
Solvent-extracted meal, 1989 crop	538	485	212	238.7 (44%)
	511	492	204	238.7 (47%)
	538	517	260	293.0 (54%)
Screw-pressed meal, 1989 crop (single-pressing)	684	705	505	568.6 (83%)
Screw-pressed meal, 1989 crop	494	478	320	359.6 (73%)
(double-pressing)	272	268	114	100.4 (37%)

[a] All values are averages of two 2 g replicates.

sion line ($y = 11.8 + 0.982x$) could be used as an estimate of overall recovery (98.2 ± 3.3%) in the analysis of gossypol in cottonseed meal and mixed feed samples.

The precision of the method was studied by assaying, on each of 3 different days, 6 samples from a mixed feed containing cottonseed meal. To estimate the overall precision, the raw data (Table 3) were subjected to analysis of variance and expected mean squares for the 1-way classification-balanced design (53). Within-day precision was 4.3%, between-day precision was 5.1%, and overall precision was 6.7%.

The proposed method, although originally developed for the determination of unbound and acetone-soluble bound gossypol, could also distinguish the unbound and the hydrophilic and lipophilic forms of soluble bound gossypol on the basis of their different solubilities in aqueous acetone and chloroform. Such a discrimination, although it cannot be considered highly accurate, might be extremely important in evaluating the toxicity of cottonseed meal. Evidence has been presented (3, 54) that the lipophilic form, presumably a gossypol–phospholipid complex, is approximately equivalent to the unbound gossypol in physiological activity, whereas the hydrophilic form, presumably a gossypol amino acid or peptide complex, is not. The results obtained for various cottonseed meal samples showed that unbound gossypol constituted 30.9–37.1% of the total gossypol content in fresh solvent-extracted meals (Table 4) and 16.4–24.5% or 9.0–15.3% in stocked solvent-extracted or screw-pressed meals, respectively. The lipophilic and hydrophilic forms of bound gossypol averaged 42.5 and 31%, respectively, of the gossypol content of all samples analyzed. Although these findings cannot be compared with those of a pertinent experiment based on the AOCS method and paper chromatography (3, 54) because of the different quantitation systems used, it may be noted that the free gossypol content of an expeller-processed cottonseed meal sample was found by the AOCS method to be composed of 40% lipophilic and 60% hydrophilic gossypol.

The sum of lipophilic and hydrophilic forms is what is called soluble bound gossypol (Table 5). To verify the values found for soluble bound gossypol by another method, a second-derivative spectrophotometric method (46), similar in sample handling to the LC method without ascorbic acid, was used. Free gossypol content of all samples was determined by directly applying this method; the sum of unbound gossypol and gossypol-related compounds was established by applying the method without the hydrolysis step, as in LC analysis for unbound gossypol. Soluble bound gossypol was calculated by subtracting the concentrations found. The results obtained (Table 5), averaging 11% lower, were in remarkably good agreement with those by the LC method.

Free gossypol values determined by the derivative method were much higher than the unbound and acetone-soluble bound gossypol values obtained by the proposed method. To evaluate this inconsistency, all samples were also analyzed by both the AOCS method (4) and the recently published LC method (47) for free gossypol determination. The data presented in Table 6 show that the proposed method gives values comparable with those of the LC method, whereas the AOCS method gives higher values that are in agreement with the derivative method. The disagreement found between the recently published LC method and the AOCS method is rather surprising in view of the earlier reported (47) agreement between these methods.

The data in Table 6 also suggested that 37–83% of the AOCS free gossypol was actually gossypol *per se* derived from unbound and acetone-soluble bound gossypol. These findings are not entirely unexpected considering the numerous molecular changes that gossypol undergoes by the action of heat and air oxygen during cottonseed processing. The explanation is that the AOCS method measures unbound, acetone-soluble bound, and various transformation and/or degradation products of gossypol having an aldehyde moiety, whereas the proposed method measures only unbound and acetone-soluble bound

gossypol. This is also the reason why standard solutions of gossypol in methanol after storage at 37°C for 29 days, although negative to gossypol by LC, gave erroneous positive readings of up to 12% of the original concentration by aniline reaction-based methods (38).

The toxicity of transformation and/or degradation products of gossypol has been little studied. According to the only paper dealing with this subject (55), the product obtained by heating crystalline gossypol in air has no pronounced toxicity; doses of 400–800 mg/kg body weight did not show any toxic effect when introduced orally into rabbits. On the other hand, there is no complete agreement among researchers as to the exact level of free gossypol that impairs animal performance; feeding tests on rats and poultry have shown that the toxicity of cottonseed meals cannot always be explained on the basis of their free gossypol content as determined by reaction-based methods (26–29). Therefore, the developed LC method might be valuable as an alternative to the AOCS method in investigating the toxicity of cottonseed meals.

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MYCOTOXINS

Immunoassay of Ochratoxin A, Using Antibodies Developed Against a New Ochratoxin–Albumin Conjugate

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A derivative of ochratoxin A was linked to bovine serum albumin in such a way that the carboxylic group of ochratoxin A was left unmodified. Lysine was substituted for phenylalanine in ochratoxin A, and the ε-amino group was linked to the protein. The conjugate was injected into 2 rabbits; antibodies against ochratoxin A were developed and used to develop an indirect competitive enzyme-linked immunosorbent assay. The detection limits for ochratoxin A in incubation buffer were 0.07 and 0.02 ng ochratoxin A/mL with the 2 antisera, respectively. Three hundred human plasma samples were purified by a novel sample preparation method and were analyzed by the immunosorbent assay. The detection limits for ochratoxin A in plasma samples were 0.2 and 0.1 ng ochratoxin A/mL with the 2 antisera, respectively. The cross-reactivity of the 2 anti-ochratoxin A sera was high for the ochratoxin A methyl ester, about 20% for ochratoxin C, and low for (4*R*)-4-hydroxyochratoxin A, ochratoxin α, ochratoxin B, and 4-hydroxyochratoxin B. No cross-reactivity was seen for phenylalanine and lysine.

Ochratoxin A (Figure 1a) is a secondary metabolite of *Penicillium* and *Aspergillus* species. It is commonly found in both human food and animal feed, as well as in human and swine blood after ingestion. Ochratoxin A is a mycotoxin with carcinogenic, nephrotoxic, teratogenic, and immunotoxic properties. The major target for its toxicity in mammalian species is the proximal convoluted tubules in the kidneys (for review see Kuiper-Goodman and Scott [1]).

Several methods for the detection of ochratoxin A have been developed over the years. Thin-layer chromatography (TLC) (2) and liquid chromatography (LC) (3–5) are 2 common techniques for the analysis of ochratoxin A. Unfortunately, these methods can be time-consuming. An enzyme-linked immunosorbent assay (ELISA) presents the possibility of high sample capacity and could, therefore, be useful in screening programs. The development of antibodies against ochratoxin A and the use of the ELISA technique has been described in the literature (6–10). All antibodies used in these assays have been generated, using the same approach as Aalund et al. (11) and Chu et al. (12), against an ochratoxin A–protein conjugate with the toxin linked through its carboxylic group to the protein (Figure 1c). In this study, we have used a modified ochratoxin A conjugate for the generation of antibodies. Lysine was substituted for phenylalanine in ochratoxin A, and the ε-amino group was linked to bovine serum albumin (BSA) (Figure 1b). The prepared antibodies were used to develop an indirect competitive ELISA for the determination of ochratoxin A in plasma samples.

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Experimental

Apparatus

(a) *ELISA reader.*—Easy reader, EAR 340 AT, SLT-labinstruments, Austria.

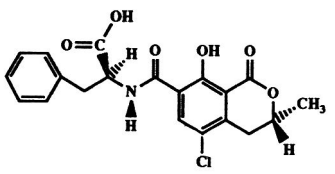
(b) *ELISA reader.*—Titertek Uniskan Type 360, EFLAB OY, Finland.

(c) *Microtiter plates.*—Nunc Immunoplate Maxisorp, A/S Nunc, Denmark.

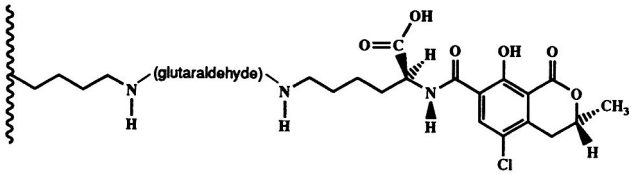
(d) *Liquid scintillation analyzer.*—Packard liquid scintillation analyzer, Tri-Carb 1550, Canberra-Packard International, Switzerland.

Reagents

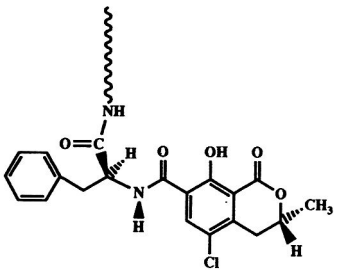
(a) *Chemicals.*—Ochratoxin A and C were produced according to Fuchs et al. (13). Ochratoxin B was a generous gift from Michael E. Stack, U.S. Food and Drug Administration, Washington, DC. (4*R*)-4-hydroxyochratoxin A and 4-hydroxyochratoxin B were generous gifts from Fredrik C. Størmer, National Institute of Public Health, Oslo, Norway. Ochratoxin A methyl ester was prepared by esterification in methanol with 10% boron trifluoride (5). [^{14}C]Ochratoxin A was prepared as described by Breitholtz-Emanuelsson et al. (14). BSA, anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate, and phosphatase substrate tablets (*p*-nitrophenyl phosphate) were purchased from Sigma Chemical Co., St. Louis, MO. Freund's complete adjuvant was purchased from Difco Laboratories, Detroit, MI. L-[4,5-^{3}H] lysine monohydrochloride was purchased from Amersham International plc, United Kingdom. Ready Safe, Beckman, USA, scintillation cocktail was used. Cyanogen bromide-activated sepharose was purchased from Pharmacia, Uppsala, Sweden. All water used was distilled and



a) Ochratoxin A



b) Oα-lys-alb



c) OA-alb

Figure 1. The structure of ochratoxin A and 2 ochratoxin–protein conjugates: (a) Ochratoxin A; (b) lysine substituted for phenylalanine in ochratoxin A and the ε-amino group of lysine linked to bovine serum albumin (Oα–lys–alb) [the mechanism of the coupling reaction of glutaraldehyde is not completely understood; therefore, the linkage between Oα–lys and alb is simply indicated by -(glutaraldehyde)-]; and (c) ochratoxin A linked through its carboxylic group to bovine serum albumin (OA–alb).

run through a MilliQPLUS (Millipore Corp., Bedford, MA), system. All other chemicals used were of pro analysis grade and purchased from Merck-Schuchardt & Co., Germany.

(**b**) *Sodium phosphate buffer, 0.1M.*—0.1M NaH_2PO_4–Na_2HPO_4 buffer at pH 7.0.

(**c**) *Sodium phosphate buffer, 10mM.*—10mM NaH_2PO_4–Na_2HPO_4 buffer at pH 7.0.

(**d**) *Sodium phosphate buffer, 5mM.*—5mM NaH_2PO_4–Na_2HPO_4 buffer at pH 8.0.

(**e**) *Equilibration buffer.*—10mM NaH_2PO_4–Na_2HPO_4 buffer at pH 7.5, 0.5M NaCl.

(**f**) *Rinsing buffer.*—0.1M sodium acetate–acetic acid buffer at pH 4, 0.5M NaCl.

(**g**) *Coating buffer.*—50mM $NaHCO_3$–Na_2CO_3 buffer at pH 9.6.

(**h**) *Washing solution.*—0.9% (w/v) NaCl, 0.05% (v/v) Tween 20.

(**i**) *Incubation buffer.*—50mM KH_2PO_4–K_2HPO_4 buffer at pH 7.5, 150mM NaCl, 0.05% (v/v) Tween 20.

(**j**) *Enzyme substrate buffer.*—1M diethanolamine, 0.5mM $MgCl_2$; pH 9.8 adjusted with 1M HCl.

(**k**) *Enzyme substrate.*—One phosphatase substrate tablet (5 mg) dissolved in 5 mL enzyme substrate buffer, prepared just before use.

(**l**) *Potassium phosphate buffer, 1M.*—1M KH_2PO_4–K_2HPO_4 buffer at pH 8.0.

(**m**) *Plasma standard.*—Plasma standards were prepared by adding known amounts of a stock solution of ochratoxin A to portions of pooled plasma. The stock solution (10^{-4}M ochratoxin A in Tris–H_2SO_4–buffer at pH 7.50; Tris = tris(hydroxymethyl)-aminomethane) was calibrated spectrophotometrically at 380 nm, using the extinction coefficient 5680 $M^{-1}cm^{-1}$ (15).

Preparation of Modified Ochratoxin–Protein Conjugate (Oα–lys–alb)

Preparation of ochratoxin α from ochratoxin A.—Ochratoxin α was prepared by acid hydrolysis of ochratoxin A as described by van der Merwe et al. (16), but without purification by sublimation. The product was purified on a preparative TLC plate in the chromatographic system toluene–dioxane–formic acid (95 + 35 + 4, v/v). Ochrotoxin α was extracted from the gel with acetone–formic acid (99 + 1, v/v). The solvent was evaporated under reduced pressure.

Linkage of lysine to BSA.—Lysine was linked to BSA with glutaraldehyde as a coupling agent, as described by Peskar et al. (17), for the coupling of normetanephrine to a polypeptide. BSA, 200 mg (2.9 μmol), and L-lysine monohydrochloride, 106 mg (580 μmol), were dissolved in 20 mL 0.1M sodium phosphate buffer. To facilitate determination of the amount of lysine conjugated to albumin, 1 μCi L-[4,5-^{3}H] lysine monohydrochloride (75 Ci/mmol) was added to the mixture. Then, 10 mL 21mM glutaraldehyde in 0.1M sodium phosphate buffer was added dropwise to the mixture with continuous stirring. The mixture was incubated 2 days at room temperature with continuous stirring. The reaction was stopped by the addition of 1 mL 1M lysine in 0.1M sodium phosphate buffer. The mixture was incubated for an additional hour before dialysis against 10mM sodium phosphate buffer. The ratio of lysine to albumin as determined by liquid scintillation counting and absorbance measurements was found to be 13:1.

Linkage of ochratoxin α to the lysine–BSA conjugate.—Ochratoxin α (10.3 mg, 40 μmol) was dissolved in 250 μL dimethyl formamide (DMF). The solution was cooled to 12°C, and 6 μL triethylamine (43 μmol) and 6 μL isobutyl chloroformate (46 μmol) were added to the ochratoxin α solution. The mixture was kept at 12°C for 2 h. The precipitate formed was centrifuged to the bottom of the tube, and the supernatant was added to 2.7 mg solid NaN_3 (42 μmol). The mixture was kept at room temperature for 30 min. Part of the ochratoxin α azide formed (169 μL, 26 μmol) and 5 μL (36 μmol) triethylamine were added to 3 mL lysine–BSA conjugate (0.26 μmol BSA).

The mixture was incubated with continuous stirring for 45 h at 4°C. The solution was dialyzed against 5mM sodium phosphate buffer. The product was analyzed spectrophotometrically to determine the ratio of modified ochratoxin to albumin. The spectrum from 200 to 330 nm was measured, and the albumin concentration was determined at 208 nm by using a calibration curve of albumin. Two spectra were measured between 330 and 430 nm, one with the sample in an acidic solution and the other with the sample in an alkaline solution. The modified ochratoxin concentration was determined at 380 nm by taking the absorbance difference between the acidic (HCl, pH 3) and the alkaline (NaOH, pH 9) spectrum, using the extinction coefficient 5680 $M^{-1}cm^{-1}$ (15).

Immunization

Two rabbits, A and B, were used for the production of antisera. For the first immunization, 0.6 mL Oα–lys–alb conjugate (3.1 mg conjugate/mL) with the ochratoxin–albumin ratio 15:1 was emulsified with 1 mL Freund's complete adjuvant. The rabbits were injected subcutaneously at the thigh with 0.5 and 0.3 mL of the mixture, respectively. After 28 days, the rabbits received a booster injection, subcutaneously at the thigh, using a conjugate (4.9 mg conjugate/mL) with the ratio 9:1. No Freund's adjuvant was used this time. The animals were injected with 0.5 and 0.4 mL conjugate, respectively. After another 2 months, a second booster was administered. One mL Oα–lys–alb conjugate (5.3 mg conjugate/mL, ratio 10:1) was emulsified with 0.5 mL Freund's complete adjuvant. The rabbits were both given 0.5 mL of the mixture in the muscle of the thigh.

Purification of Antibodies from Sera

Ten days after the last booster, the rabbits were bled and the sera collected. Antibodies with affinity against BSA were removed from the sera by BSA–sepharose affinity chromatography. The antibodies with affinity against ochratoxin A did not bind to the column. BSA was linked to cyanogen bromide-activated sepharose according to the manufacturer's directions. A small column of BSA–sepharose (5 mL) was packed. The column was equilibrated with equilibration buffer, and 0.5 mL serum was applied to the column. The antibodies with affinity to ochratoxin A were eluted in 1 mL portions with equilibration buffer. The absorbances of the eluted portions were measured at 280 nm, and the fractions with a high absorbance were tested in an indirect ELISA to determine their antibody titers. Fractions with the same titer were pooled, yielding 8 mL diluted antibodies per rabbit. Before the next run, the column was cleaned from attached antibodies with rinsing buffer and equilibrated with equilibration buffer. The purified antibodies were stored at –20°C until used.

ELISA Procedures

Coating of ELISA plates.—The immunoplates (Nunc Immunoplate Maxisorp) were coated with the Oα–lys–alb conjugate (5.3 mg conjugate/mL). The ratio of modified ochratoxin to albumin was 10:1. The conjugate was diluted in coating buffer to the albumin concentration, 0.01 μg/mL, and 200 μL of this diluted conjugate was added to each well of the immunoplate. The plates were covered with laboratory film (parafilm) and incubated overnight at 4°C.

Indirect ELISA.—The indirect ELISA was used for the determination of the antibody titer against ochratoxin A. The coated plates were washed 3 times with washing solution. The purified antibodies were diluted in incubation buffer in a dilution series, and 100 μL of the respective dilution was added to the appropriate well of the Oα–lys–alb coated plates. The samples were run in triplicate. The plates were incubated 2 h at room temperature and washed 3 times with washing solution; then, 100 μL anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate diluted 1 + 2000 in incubation buffer was added, and the plates were incubated 1 h at room temperature. The plates were washed 3 times, and 100 μL freshly prepared enzyme substrate was added to each well. The plates were incubated in darkness at room temperature for 40 min, and the absorbance was measured at 405 nm. The titers were determined at 60% of the maximum absorbance.

Indirect competitive ELISA.—An indirect competitive ELISA was used to determine ochratoxin A in human plasma samples and to determine the antibody specificity. Immuno plates were coated and washed as described above. A 50 μL sample (see *Preparation of Plasma Samples*), ochratoxin A standard, or, for the cross-reactivity experiments, the competing substance was added to appropriate wells. Immediately afterward, 50 μL antiserum, diluted in incubation buffer, was added to each well. The purified antiserum of rabbit A was diluted 1 + 1000, and that of rabbit B was diluted 1 + 400. The total protein concentrations in the dilutions were 3 and 7 μg/mL, respectively. The assay plates were incubated 2 h at room temperature and thereafter treated as described for the indirect ELISA. In the cross-reactivity study, the competing substances were diluted in incubation buffer. For plasma samples, 2 types of standard curves of ochratoxin A were run: (*1*) ochratoxin A diluted in incubation buffer, and (*2*) spiked plasma samples purified for the ELISA.

Preparation of Plasma Samples

Three hundred microliters of plasma and 400 μL acetone were added to an Eppendorf tube and mixed by vortexing, and the tube was centrifuged in an Eppendorf centrifuge at 10 000 × *g* for 2 min. The supernatant was removed from the protein precipitate to a new Eppendorf tube. To the supernatant, 100 μL 1M potassium phosphate buffer at pH 8.0 and 500 μL chloroform were added. The solution was mixed by vortexing for 10 s, and the tube was centrifuged at 10 000 × *g* for 5 min. The upper aqueous layer was used as sample in the indirect competitive ELISA.

Recovery

The recovery was determined by adding ^{14}C-labeled ochratoxin A to spiked plasma samples. To samples spiked with 1 and 0.1 ng ochratoxin A/mL, 0.09 nCi ^{14}C-ochratoxin A (396 Ci/mol) was added. To samples spiked with 100, 10, and 0 ng ochratoxin A/mL, 0.99 nCi of the labeled ochratoxin A was added. After the addition of the labeled ochratoxin, the plasma

samples were treated as described under *Preparation of Plasma Samples*. Two hundred microliters of the aqueous phase was mixed with 10 mL scintillation cocktail, and the radioactivity was measured by liquid scintillation counting.

Analysis of Human Plasma Samples by LC

To determine whether human plasma samples contained ochratoxin A, 125 human plasma samples collected both in Visby, Sweden, and in a Balkan endemic nephropathy village in Yugoslavia were analyzed by LC. The samples were purified and analyzed by the method described by Breitholtz et al. (4).

Results

ELISA

The detection limits of ochratoxin A were determined by using an absorbance value 15% beneath the plateau of the standard curves. The detection limit of ochratoxin A in incubation buffer was 0.07 ng ochratoxin A/mL (3.5 pg ochratoxin A/well) with the purified antiserum from Rabbit A and 0.02 ng ochratoxin A/mL (1 pg ochratoxin A/well) with antibodies produced by Rabbit B. For ochratoxin A in incubation buffer and plasma samples treated as described under *Preparation of Plasma Samples*, the detection limits were 0.2 and 0.1 ng/mL with the 2 antisera, respectively. For the plasma samples analyzed in Zagreb (see *Analysis of Human Plasma Samples*), the detection limit was 0.5 ng ochratoxin A/mL plasma.

The recoveries in the sample preparation step for 0.4, 1.3, 3, 13, and 103 ng ochratoxin A/mL were 53, 56, 62, 61, and 59%, respectively.

The cross-reactivities of the antibodies toward other ochratoxin derivatives, shown in Table 1, were calculated as the amount of ochratoxin A required for 50% inhibition divided by the amount of competing substance required for 50% inhibition. The ochratoxin A concentrations causing 50% inhibition were 0.4 and 0.2 ng/mL with the 2 antisera, respectively.

Analysis of Human Plasma Samples

A total of 210 human plasma samples were collected in endemic and nonendemic villages in Yugoslavia. These samples were analyzed in Zagreb, Yugoslavia, using a Titertek Uniskan type 360 ELISA- spectrophotometer for measuring the absorbance. In 58% of these samples, the ochratoxin A contamination level was <0.5 ng/mL, 26% of the samples were contaminated in the concentration range 0.5–1 ng/mL, and 16% of the samples were contaminated with >1 ng/mL.

Correlation Between ELISA and LC Analyses

Thirty-five samples from an endemic village in Yugoslavia and 90 samples collected in Visby, Sweden, were analyzed by the ELISA method in Stockholm, Sweden, using the EAR 340 AT ELISA-reader for measuring the absorbance. The samples from Visby had already been analyzed by LC, with a 0.3 ng ochratoxin A/mL plasma detection limit (4). The samples from Yugoslavia were analyzed by LC, with a 0.02 ng ochratoxin A/mL plasma detection limit, and the results from the LC analyses and the ELISA analysis were compared (Figure 2). A straight line through the points, fitted by the least squares method, had a slope of 0.98, showing a perfect agreement between the 2 methods. No false-positive or false-negative samples were found when the results from the ELISA and LC analyses were compared at levels 3 times the detection limit of the methods.

Table 1. Cross-reactivities of the 2 polyclonal antiochratoxin A sera[a]

Substance	Antiserum, %	
	Rabbit A	Rabbit B
Ochratoxin A	100	100
Ochratoxin A methyl ester	65	45
Ochratoxin C	20	15
(4*R*)-4-Hydroxyochratoxin A	5	3
Ochratoxin α	3.5	1.5
Ochratoxin B	0.2	0.08
4-Hydroxyochratoxin B	0.3	0.2
Phenylalanine	<0.001	<0.001
Lysine	<0.001	<0.001

[a] The cross-reactivities were calculated as the amount of ochratoxin A required for 50% inhibition divided by the amount of competing substance required for 50% inhibition.

Discussion

Because of the low molecular weight of ochratoxin A, the molecule does not stimulate the immune system or antibody production on its own. Therefore, ochratoxin A must be linked to a higher molecular weight carrier, e.g., a protein, to induce the immune system. There are many reports describing the development of antibodies against ochratoxin A. All these methods have used an ochratoxin A–protein conjugate with the toxin linked through its carboxylic group to a protein or polypeptide (Figure 1c, OA–alb). In this study, antibodies against ochratoxin A were generated against a novel ochratoxin–protein conjugate (Figure 1b, Oα–lys–alb). Lysine was substituted for phenylalanine in ochratoxin A, and the ε-amino group was linked to BSA, leaving the carboxylic group of ochratoxin A unmodified. This free carboxylic group is important for the charge distribution, as it is dissociated and charged at the pH used for analysis. We calculated the electropotential surfaces of ochratoxin A and of the protein conjugates using the molecular modelling program SYBYL (18). We found that the electropotential surface of the isocoumarin part of the modified ochratoxin A–protein conjugate (Oα–lys–alb) and the electropotential surface of ochratoxin A were virtually identical, whereas that of the ochratoxin A–protein conjugate (OA–alb) was very different. The similarity in charge distribution of the antigen and the analyte may explain why the present polyclonal antibodies showed high affinity while our earlier attempts, using antigens with masked carboxylic groups, induced low affinity antibodies.

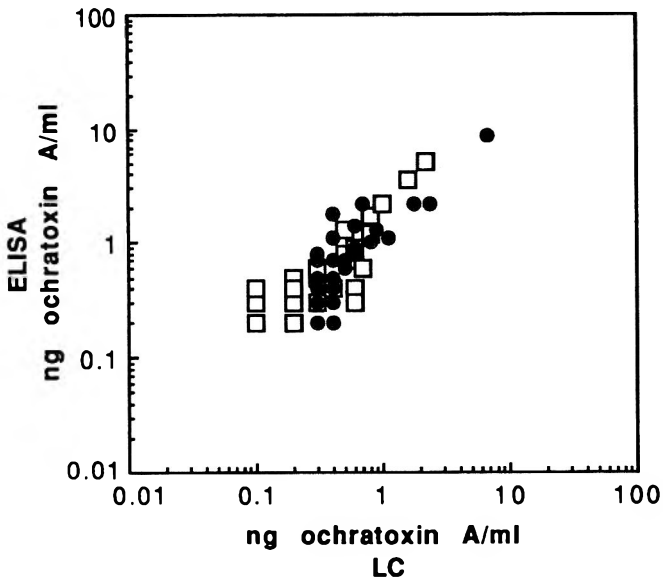


Figure 2. Correlation between ELISA and LC analyses of human plasma samples. For the samples from Visby, Sweden, (•), the detection limits of the ELISA and LC analyses were 0.2 and 0.3 ng ochratoxin A/mL plasma, respectively. For the samples from an endemic village in Yugoslavia, (□), the detection limits were 0.2 and 0.02 ng ochratoxin A/mL plasma by the 2 techniques, respectively. A straight line through the points, fitted by the least squares method, has a slope of 0.98.

The detection limit of ochratoxin A in buffer using the polyclonal antibodies produced in this study is lower than earlier reported detection limits (6, 19). The detection limit, determined at 15% inhibition, is comparable to the detection limit determined at 10% inhibition by Kawamura et al. (7), using their best monoclonal antibody. Considering the difference in the determination of the detection limit, the polyclonal antibodies produced in this study yield a lower detection limit than the monoclonal antibody produced by Kawamura et al. (7).

An essential part of an immunoassay should be to have an easy sample cleanup and preparation step. If the sample preparation is time-consuming, the advantages of an ELISA as a rapid and efficient method are lost. The sample preparation described in this study is very fast and easy. A drawback is the low recovery, although it is constant and reproducible. With a better recovery, a lower amount of ochratoxin A in samples could be detected. A lower detection limit could also be reached through a sample concentration step, but that would decrease the efficiency considerably. Because of the low protein concentration used to coat the immunoplates in this assay, it is impossible to analyze samples in which the proteins in the sample matrix have not been removed. The proteins in the sample matrix tend to reach an equilibrium with the protein conjugate already coated in the wells. This results in replacement of the coated protein conjugate by proteins from the sample matrix, leading to interference in the assay.

The sample preparation method has been used for the purification of samples of human plasma; calf, rat, and pig sera; and milk. The whole procedure with the sample preparation and the analysis with the indirect competitive ELISA functions well with these kinds of samples and with detection limits in the 0.1 ng/g sample range.

In an immunoassay, it is impossible to make a confirmation through a derivatization step. Therefore, the confirmation has to be performed by another method. Märtlbauer and Terplan (8) have shown a good correlation between their ELISA and an LC method, using the same extraction scheme in the 2 methods. This study showed total agreement between the results from the ELISA and the LC analysis, using 2 completely different sample preparation procedures.

With few samples to analyze, the mass processing qualities of an ELISA are not used. The advantages of the ELISA technique, e.g., the large number of samples that can be analyzed daily, the use of inexpensive equipment, and the large concentration range that can be covered, are best used in epidemiological studies with thousands of samples. In such studies, the disadvantages of the logarithmic concentration scale, where the uncertainty in the concentration determination increases with the concentration, and the inability to confirm the identity of the substance are of minor importance.

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MYCOTOXINS

Liquid Chromatographic Determination of Fumonisins with 4-Fluoro-7-nitrobenzofurazan

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Fumonisins B_1 and B_2 are closely related mycotoxins produced by *Fusarium moniliforme*, *F. proliferatum*, and related species. Disadvantages of 2 fluorescence derivatizing reagents currently used for liquid chromatography (LC) are instability of the derivatives (with *o*-phthaldialdehyde–mercaptoethanol) and formation of 2 peaks (with fluorescamine). 4-Fluoro-7-nitrobenzofurazan (4-fluoro-7-nitrobenz-2-oxa-1,3-diazole; NBD-F) was, therefore, investigated. Although a larger molar excess of this reagent is required for fumonisins than for amino acids, the derivatives formed are moderately stable, and as little as 1 ng fumonisins B_1 or B_2 can be detected, with a linear response up to at least 50 ng injected. Reversed-phase LC (C_{18} column) was carried out with a mobile phase of methanol–0.05M sodium dihydrogen phosphate adjusted to pH 5 (1 + 1), which was mixed with an equal volume of acetonitrile–water (8 + 2) after 5 min. Using a modification of a strong anion exchange cleanup procedure, good recoveries (averages of 94 and 80%, respectively) of fumonisins B_1 and B_2 from ground corn, corn meal, and corn flakes in the 125–5000 ng/g range were generally obtained; the limit of detection of the overall method was about 100 ng/g. Similar results were obtained in studies with ground corn, corn meal, and corn flakes using naphthalene-2,3-dicarboxaldehyde–potassium cyanide (NDA–KCN) for derivatization; average recoveries of fumonisins B_1 and B_2 were 94 and 83%, respectively.

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Fusarium moniliforme Sheldon, a common fungal contaminant of corn, produces a variety of mycotoxins (1). Among these are the fumonisins (2), which are arousing intense interest among mycotoxicologists because of certain animal and human health problems they cause or are suspected to cause. Other *Fusarium* species are now known to produce fumonisins (3, 4). Fumonisin B_1, which has also been isolated under the name macrofusin (5), has been structurally characterized as the diester of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyeicosane (5, 6); fumonisin B_2 is the 10-deoxy analog. Two animal diseases, equine leukoencephalomalacia and pulmonary edema–hydrothorax in swine, have been associated with the occurrence of fumonisins B_1 and B_2 in the feed (7–10) and have been reproduced experimentally with purified fumonisin B_1 (10, 11). Also, a significant association between fumonisins B_1 and B_2 in corn and human esophageal cancer in southern Africa has been indicated in a preliminary study (12). Recently, fumonisin B_1 has been shown to cause liver cancer in rats (13).

Published methods for determination of fumonisins in corn use liquid chromatography (LC) or posthydrolysis gas chroma-

tography/mass spectrometry (12, 14–16). The primary amino group in the fumonisins can be derivatized for LC with fluorescence detection. However, disadvantages of 2 reagents currently used for LC are formation of 2 peaks with fluorescamine (14) and instability of the derivatives with *o*-phthaldialdehyde–mercaptoethanol (15). A number of other reagents used for fluorescence derivatization of primary amines and/or amino acids were, therefore, investigated. Of these, 4-(*N*-phthalimidyl)benzoyl chloride (phibyl chloride) (17), 4-(*N*-phthalimidyl)benzenesulfonyl chloride (phisyl chloride) (18), and 9-fluorenylmethyl chloroformate (FMOC-Cl) (19, 20) did not react with fumonisin B_1 under conditions tried; 5-(4,6-dichlorotriazinyl)-aminofluorescein (DTAF) (21) reacted very slowly and gave only a small peak; and, although 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) (22) formed a good derivative, it was not useful with corn extracts because of interferences. As a more promising reagent, 4-fluoro-7-nitrobenzofurazan (4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole; NBD-F) (23) was studied for derivatization of fumonisins and application to corn analysis. In addition, the naphthalene-2,3-dicarboxaldehyde (NDA)–potassium cyanide (KCN) reagent (24), recently used by Ware (25) for determination of fumonisin B_1 in corn, was included in the overall method.

METHOD

Safety note: Fumonisins B_1 and B_2 are suspected carcinogens and should be handled with caution.

Principle

Sample is extracted with methanol–water, and aliquot of filtered extract is cleaned up on strong anion exchange (SAX) solid-phase extraction tube. Sample extract is derivatized with NBD-F or NDA–KCN and analyzed by reversed-phase LC with fluorometric detection.

Apparatus

Equipment specified is not restrictive, and other suitable equipment may be substituted.

(**a**) *LC system.*—Altex Model 110A pumps, mixer, Beckman Analog Interface Module 406 and NEC PC-8300 controller, Shodex in-line degasser, Hewlett Packard Series 1050 autosampler, Waters Guard-Pak precolumn module (Cat. No. 88141) with Resolve C_{18} precolumn insert (Cat. No. 85824), stainless steel Ultratechsphere 5 μm ODS, 250 × 4.6 mm column (HPLC Technology, Macclesfield, UK); Kratos Spectroflow 980 fluorescence detector (ABI Analytical, Ramsey, NJ 07446); Varian (Spectra-Physics) 4270 integrator with 0.25 cm/min chart speed.

(**b**) *Shaking machine.*—Horizontal type (Arthur H. Thomas Co.).

(**c**) *Screw-cap vials.*—4 and 16 mL with Teflon liners and 2 mL autosampler vials.

(**d**) *Solid-phase extraction tubes.*—SAX columns: 1 mL Supelclean LC-SAX (Supelco Canada, Inc., 5-7007).

(**e**) *Rack.*—To support SAX columns.

(**f**) *Syringes.*—Gilson Pipetman continuously adjustable pipets P-1000 and P-200 with disposable pipet tips and Hamilton 25, 100, and 500 μL syringes.

Reagents

(**a**) *Water.*—Use distilled, deionized water throughout.

(**b**) *Solvents.*—Distilled-in-glass, methanol (LC grade for mobile phase), acetonitrile (LC grade for mobile phase), ethanol.

(**c**) *NBD-F.*—Available from Sigma Chemical Co., St. Louis, MO 63178 (Cat. No. F5883) and Molecular Probes Inc., Eugene, OR 97402 (Cat. No. F-486). Prepare 50mM solution (9.15 mg/mL) in ethanol in quantity sufficient for 1 day, and store in freezer (ca −12°C).

(**d**) *NDA.*—Available from Bioanalytical Systems, Inc., West Lafayette, IN 47906 (Cat. No. CF-1046); Molecular Probes, Inc. (Cat. No. N-1138); and Oread Laboratories, Inc., Lawrence, KS 66047 (Cat. No. N802). Dissolve 1 mg in 2 mL ethanol, and store in freezer (ca −12°C).

(**e**) *General chemicals.*—Potassium cyanide, sodium dihydrogen phosphate, sodium hydroxide, sodium borate, acetic acid, ACS grade.

(**f**) *Fumonisins B_1 and B_2.*—Available from Sigma Chemical Co. (Cat. Nos. F2643 and F3771, respectively). Prepare 125 μg/mL of each fumonisin in acetonitrile–water (3 + 1, v/v) to obtain stock solution of each fumonisin. Dilute 10–200 μL of each stock solution to 10 mL in acetonitrile–water (3 + 1, v/v) to obtain mixed working standards (125–2500 ng/mL). Store all standard solutions in freezer (ca −12°C).

(**g**) *Mobile phases for LC.*—Methanol–0.05M sodium dihydrogen phosphate adjusted to pH 5 with 2M NaOH (1 + 1, v/v) (A); acetonitrile–water (8 + 2, v/v) (B); acetonitrile–water–acetic acid (55 + 45 + 1, v/v/v) (C). Filter solutions through 47 μm diameter Millipore type FG (Fluoropore) disc, 0.2 μm, conditioned with methanol.

Sample Preparation

Grind corn or corn flakes to fine powder (20 mesh) and mix well before taking analytical sample. Coffee grinder was used for experiments reported here.

Extraction

Shake 25 g sample with 50 mL methanol–water (3 + 1, v/v) in 250 mL glass-stoppered Erlenmeyer flask for 15–30 min, and filter portion of extract through Whatman No. 1 filter paper into 16 mL vial (**Note**: Recovery studies were carried out on 0.20 scale for extraction step; consequently, extracts from 5 g samples were filtered into 4 mL vials). Remove 200 μL filtrate for SAX column cleanup step.

SAX Column Cleanup

Condition SAX column before use by washing with 2 mL methanol followed by 2 mL methanol–water (3 + 1, v/v). To speed process, apply gentle syringe pressure; let drain by gravity if column is to be used for corn flakes extract. Do not let column run dry. Add 200 μL extract to top of column and drain

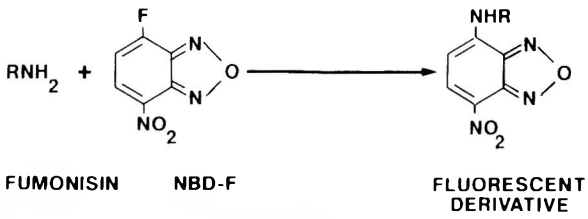

Figure 1. Reaction of NBD-F with fumonisins.

extract to frit at top of column bed. Rinse top of frit with 2–3 drops of methanol–water (3 + 1, v/v) and drain. Add 1 mL methanol–water (3 + 1, v/v) to column and drain to frit (gentle syringe pressure may be applied). Discard all of these washes. Place 2 mL vial below column to collect eluate, add 1.5 mL methanol–acetic acid (99 + 1, v/v) to top of column, and elute by gravity. Evaporate eluate carefully to dryness in heated (60°C) aluminum block and under gentle stream of nitrogen; allow last ca 0.2 mL of eluate to evaporate at room temperature.

Derivatization with NBD-F

Dissolve dried residue from cleanup step in 100 µL 0.04M sodium borate buffer, pH 8.3 (adjusted with 1N HCl) in 2 mL vial. Add 50 µL 50mM NBD-F in ethanol, preferably freshly prepared. (**Note**: NBD-F solution must be stored in freezer [−12°C] and can be used for 1–2 days.) Heat reaction mixture for 1 min in aluminum block at 60°C, and then cool with ice and add 100 µL mobile phase (A). Repeat derivatization procedure on residue obtained after evaporation at room temperature of 200 µL working standard of mixed fumonisins B_1 and B_2. Injection within 2 min after derivatization is recommended. Note that fumonisins derivatized with NBD-F are stable in mobile phase (A) up to 2 days when stored in freezer at −12°C.

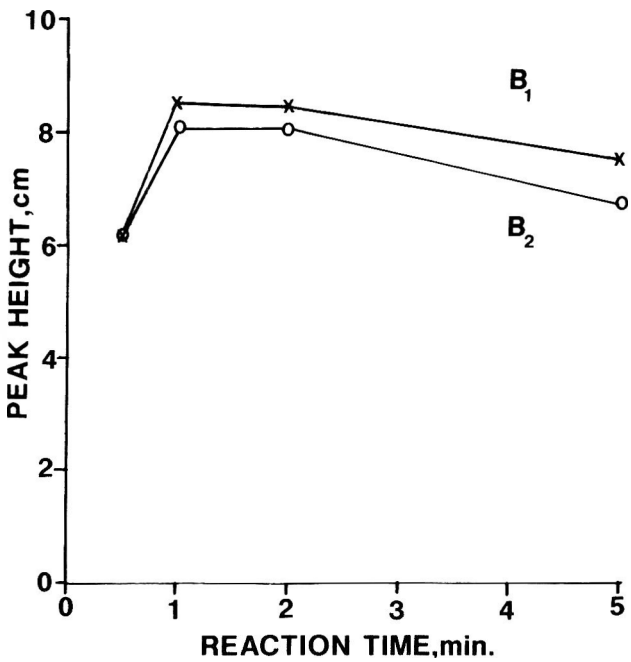

Figure 2. Effect of reaction time on derivatization of fumonisins B_1 and B_2 (30 ng each) with NBD-F at 60°C.

Derivatization with NDA–KCN

Dissolve dried residue from cleanup step in 100 µL 0.04M sodium borate buffer, pH 8.3 (adjusted with 1N HCl) in 2 mL vial. Add 50 µL 0.01M KCN and 100 µL NDA solution in methanol (0.5 mg/mL). (**Note**: NDA solution must be stored in freezer [−12°C]). Heat reaction mixture for 15 min in aluminum block at 60°C, then cool with tap water and add 250 µL mobile phase (C). Repeat derivatization on residue obtained after evaporation at room temperature of 200 µL working standard of mixed fumonisins B_1 and B_2. Note that fumonisins derivatized with NDA–KCN are stable in mobile phase (C) up to 20 h at room temperature and at least 3 days when stored in freezer at −12°C.

LC Determination

For NBD-F derivatives, use the following operating conditions: excitation wavelength, 460 nm; emission cut off, 500 nm; flow rate, 1 mL/min; mobile phases, (A) methanol–0.05M sodium dihydrogen phosphate adjusted to pH 5 with 2M NaOH (1 + 1, v/v) and (B) acetonitrile–water (8 + 2, v/v). Run 100% (A) for 5 min, and then switch to 50% (B) and 50% (A) for 15 min. At end of each day, wash column with mobile phase (B) for ca 10 min.

For NDA–KCN derivatives, use the following operating conditions: excitation wavelength, 420 nm; emission cut off, 500 nm; flow rate, 1 mL/min; mobile phases, (C) acetonitrile–water–acetic acid (55 + 45 + 1, v/v/v) and (B) acetonitrile–water (8 + 2, v/v). Run 100% (C) for 10 min, and then switch to 80% (B) and 20% (C) for additional 7 min.

Set sensitivity controls to give 30–35% of full scale response for 20 ng fumonisin injected. Chromatograph at least 3 amounts in 1–50 ng range each of fumonisins B_1 and B_2 from freshly derivatized solutions, and plot peak heights vs amounts injected to test linearity.

For determination of fumonisins, inject 25–50 µL derivatized sample extract and 25–50 µL derivatized fumonisins B_1 and B_2. Dilute sample extract if necessary with appropriate mobile phase. Compare heights of peaks from sample and standards, which must have same retention times, to obtain concentrations of fumonisins B_1 and B_2 in injected derivatized sample solution. Calculate concentrations of fumonisins in original sample as follows:

$$\text{fumonisin } B_1 \text{ } (B_2), \text{ ng/g} = \frac{XV_1V_3}{V_2W}$$

where X = concentration of fumonisin B_1 (B_2) determined above in injected derivatized sample solution (ng/mL), V_1 = volume of methanol–water (3 + 1, v/v) used for extraction, V_2 = volume of methanol–water (3 + 1, v/v) extract applied to SAX column (0.200 mL), V_3 = volume of final derivatized extract plus mobile phase added, and W = weight of sample used.

Results and Discussion

The reaction of NBD-F with fumonisins takes place at the primary amino group (Figure 1). Under conditions used by

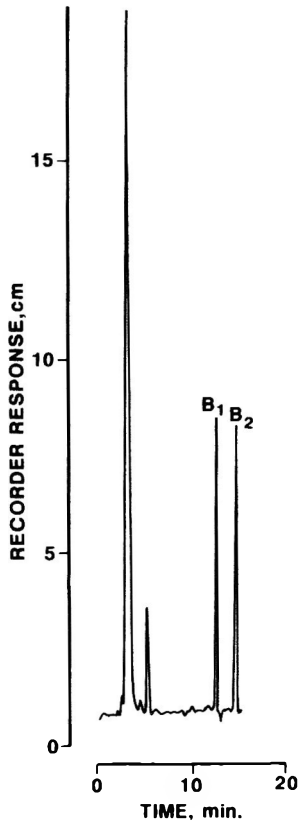

Figure 3. Liquid chromatogram of standard fumonisins B_1 and B_2 (30 ng each) after NBD-F derivatization.

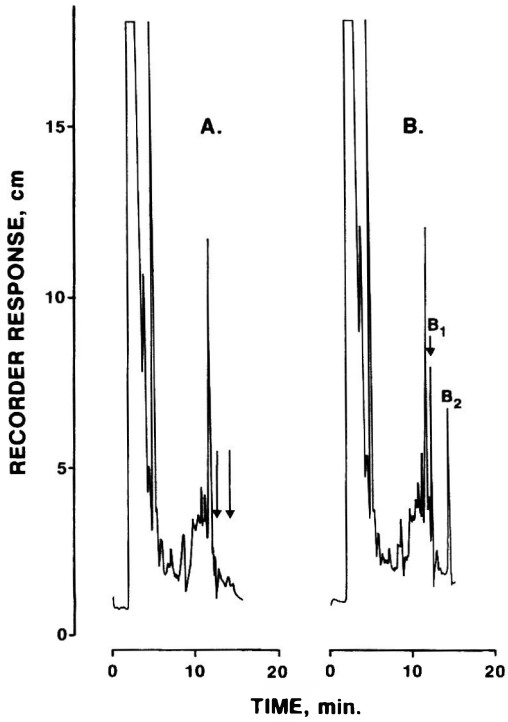

Figure 4. Liquid chromatograms of blank corn extract (A) and extract of corn spiked with 1 μg/g of both fumonisins B_1 and B_2 (B) after derivatization with NBD-F. Arrows in (A) indicate positions for fumonisins B_1 and B_2. Extract equivalent to 20 mg corn was injected.

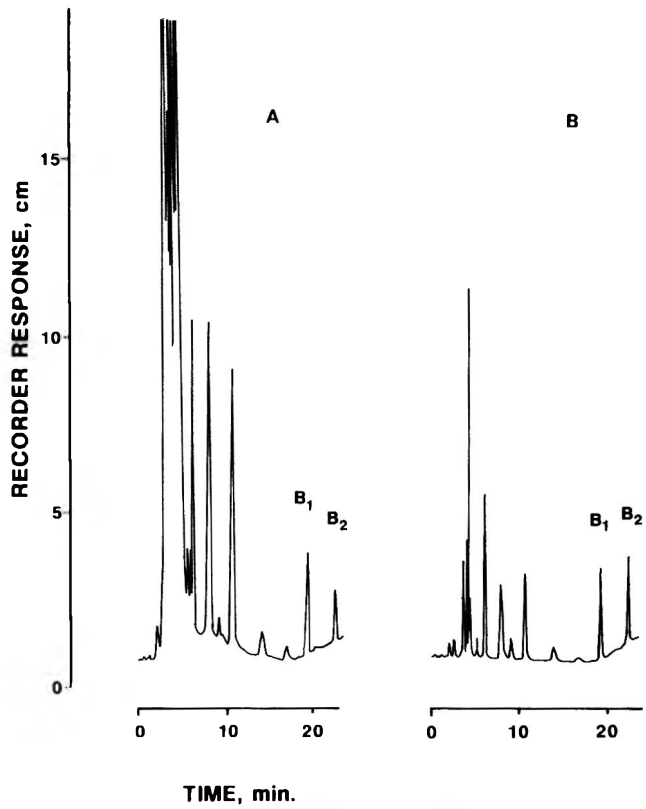

Figure 5. Liquid chromatograms of extract of corn spiked with 1 μg/g of both fumonisins B_1 and B_2 (A) and standard fumonisins B_1 and B_2 (5.5 ng each) (B) after derivatization with NDA–KCN. Extract equivalent to 5.5 mg corn was injected.

Watanabe and Imai (23) for amino acids, a somewhat larger excess of reagent was required. Thus, 2500 nmol NBD-F was needed for up to 0.69 nmol (500 ng) of each fumonisin, whereas Watanabe and Imai (23) reported that a total of 10 nmol of amino acids could be derivatized with 1000 nmol NBD-F. As previously found for amino acids (23), a reaction time of 1 min at 60°C was observed to be sufficient for the fumonisins (Figure 2). LC determination of the fumonisin NBD-F derivatives showed approximately equal fluorescence response for fumonisins B_1 and B_2 (Figure 3). The standard curves were completely linear at least up to 50 ng fumonisin B_1 or B_2 injected, and as little as 1 ng could be detected. Stability of the fumonisin NBD-F derivatives was better than those formed with *o*-phthaldialdehyde–mercaptoethanol (15) but depended on reagent quality. In the LC mobile phase (A) at room temperature, peak heights did not start to decrease until after 30 min in one experiment, but half-lives as low as 20 min have been observed.

The NBD-F derivatization reaction was applied to determination of fumonisins B_1 and B_2 in ground corn and corn meal. The extract was cleaned up by SAX solid-phase extraction, previously used for fumonisins by Shephard et al. (15). Their procedure was modified by using a 1 mL instead of a 3 mL solid-phase extraction tube. This allows considerable reduction in volumes of sample extract solution and other solvents applied to the column so that gravity elution is feasible; also, the

Table 1. Recoveries of fumonisins B_1 and B_2 from ground corn, corn meal, and ground corn flakes[a]

Sample	Extn time, min	Derivatizing reagent	Mean rec. (± SD), %							
			125 ng/g		250 ng/g		1000 ng/g		5000 ng/g	
			B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2
Corn	15	NBD-F	100	120	115	100	111	103	84	76
			(n = 1)		(n = 2)		(n = 2)		(n = 2)	
	15	NDA–KCN	80	100	80	75	92[b]	74[b]	78[b]	69[b]
			(n = 1)		(n = 1)		(n = 1)		(n = 1)	
	30	NDA–KCN	—	—	89 (± 19)	100 (± 21)	83 (± 3)	63 (± 3)	—	—
			—		(n = 3)		(n = 3)		—	
Corn meal	15	NBD-F	—	—	114 (± 12)	96 (± 5)	—	—	80 (± 6)	74 (± 7)
			—		(n = 4)		—		(n = 4)	
	15	NDA–KCN	100	100	120	83	—	—	—	—
			(n = 1)		(n = 1)		—		—	
Corn flakes	15	NBD-F	—	—	84 (± 14)	62 (± 27)	73 (± 2)	43 (± 9)	—	—
			—		(n = 3)		(n = 3)		—	
	30	NDA–KCN	—	—	100 (± 9)	73 (± 5)	103 (± 19)	95 (± 10)	—	—
			—		(n = 4)		(n = 4)		—	

[a] After corrections where necessary for just detectable fumonisin B_1 equivalents in blank corn and corn meal.
[b] Determinations made after repeat cleanup of an aliquot of the same corn extract used for NBD-F results.

methanol wash, which resulted in some loss of fumonisin B_2, was omitted. Figure 4 shows chromatograms corresponding to blank and spiked corn analyzed by this method with NBD-F derivatization. Table 1 includes satisfactory method recoveries at 125–5000 ng/g spiking levels, although there appears to be a matrix effect for fumonisin B_2 in corn flakes. For these experiments, the shaking time for extraction of the samples was 15 min, which appeared to be sufficient for spiked samples. The detection limit was about 100 ng/g (signal:noise ratio, 3:1).

While the work on NBD-F derivatization was in progress, G. Ware (Conference on Fumonisins and *Fusarium moniliforme*, September 6–7, 1990, Ames, IA) reported on the use of NDA–KCN for derivatization of fumonisins. This procedure resulted in derivatives that were more stable than the NBD-F derivatives, and the reagent was included in the method described here. Good results were obtained, as shown by the chromatograms in Figure 5 and the recoveries included in Table 1. Detection limits for fumonisins B_1 and B_2 in corn and corn meal were about 100 ng/g (signal:noise ratio, 3:1). Retention times for the fumonisin NDA–KCN derivatives can be shortened, if required, by decreasing the time for running the acetonitrile–water–acetic acid (55 + 45 + 1, v/v/v) mobile phase (C) and then switching to 100% acetonitrile–water (8 + 2, v/v) (B). Comparison of the 2 derivatizing reagents for analysis of the same extract of a "naturally" contaminated (field-inoculated) corn sample for fumonisins B_1 and B_2 gave, respectively, 1.7 and 0.50 µg/g using NBD-F, and 1.8 and 0.35 µg/g using NDA–KCN.

In conclusion, NBD-F is a useful alternative fluorescence derivatizing reagent for determination of fumonisins B_1 and B_2 in corn, although NDA–KCN is preferred for routine use.

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MYCOTOXINS

Liquid Chromatographic Determination of Fumonisins B_1 and B_2 in Corn and Corn Products

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A liquid chromatographic (LC) method is described for determining fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2), metabolites of *Fusarium moniliforme*, in corn and corn products. The metabolites are extracted with methanol–water (3 + 1), and the extract is added to a disposable strong anion exchange column. After the FB_1 and FB_2 are eluted from the column with methanol–acetic acid (199 + 1) and the eluate is evaporated to dryness, the metabolites are derivatized with *o*-phthaldialdehyde and 2-mercaptoethanol to make highly fluorescent derivatives. Reversed-phase LC with fluorescence detection is used for the final separation and determination. The estimated limit of quantitation is 10 ng/g for each metabolite. The average recoveries from corn were 67% for FB_1 and 71% for FB_2, with relative standard deviations of 5% for FB_1 and 3% for FB_2. The identities of the fumonisins were confirmed by mass spectrometry. Applications of the method include the analyses of corn meal and breakfast cereals.

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Fusarium moniliforme, one of the most common molds associated with corn, is known to produce a variety of mycotoxin metabolites, including the recently identified fumonisins B_1 (FB_1) and B_2 (FB_2) (1). The fumonisins have been shown to cause the disease leukoencephalomalacia (LEM) in horses (2) and porcine pulmonary edema (PPE) in swine (3). *F. moniliforme* has been suggested as a cause of rickets like deformities in chickens and of the increased incidence of human esophageal cancer in Transkei, South Africa, and in Lin Xian, The People's Republic of China (4). An increased incidence of LEM and PPE in all regions of the United States was associated with the 1989 corn crop.

For most of the presently used methods for the determination of fumonisins in corn, the lower limit of quantitation is

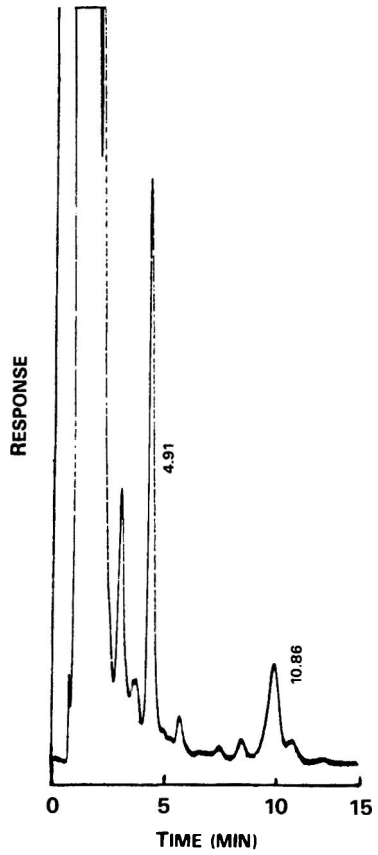

Figure 1. Liquid chromatogram of OPA derivatives of fumonisins B_1 (4.91 min, 138 ng/g) and B_2 (10.86 min, 62 ng/g) extracted from naturally contaminated grits. Mobile phase, acetonitrile–water–acetic acid (50 + 50 + 1); flow rate, 1 mL/min; column, μBondapak C_{18}, 3.9 × 150 mm; and fluorescence detector, 335 nm excitation and 440 nm emission.

around 1 μg/g (5, 6). To determine the occurrence of FB_1 and FB_2 in corn-based human foods, a method was needed with an even lower limit of quantitation. One liquid chromatographic (LC) method (6) that gave satisfactory results was modified in our laboratory to make the procedure more rapid. We evaluated the modified method, using naturally contaminated corn and spiked controls. This paper describes the modified method and presents the results of our analyses of corn and corn-based food.

METHOD

Apparatus

(a) *Explosion-proof blender.*—Waring Model EP-1 with 500 mL jar and cover.

(b) *Strong anion exchange (SAX) column.*—Bond-Elut SAX, 3 mL capacity with 500 mg sorbent (Analytichem, Harbor City, CA 90710).

(c) *Vacuum apparatus.*—Solid-phase extraction manifold (Supelco, Bellefonte, PA 16823).

(d) *LC system.*—Model 6000A pump, U6K injector, and μBondapak C_{18} column, 3.9 × 150 mm (Millipore-Waters, Milford, MA 01757); RF-535 fluorescence detector (Shimadzu, Columbia, MD 21046); and SP-4100 integrator (Spectra-Physics, San Jose, CA 95134).

(e) *Heating module.*—Reacti-Therm (Pierce Chemical Co., Rockford, IL 61105).

Reagents

(a) *Solvents.*—Acetonitrile, methanol, and acetic acid (all ACS grade).

(b) *LC mobile phase.*—Acetonitrile–water–acetic acid (50 + 50 + 1).

(c) *Sodium tetraborate.*—ACS grade.

(d) *o-Phthaldialdehyde (OPA) and 2-mercaptoethanol.*—Aldrich Chemical Co., Inc., Milwaukee, WI 53201.

(e) *OPA reagent.*—Dissolve 40 mg OPA in 1 mL methanol and dilute with 5 mL 0.1M sodium tetraborate. Add 50 μL 2-mercaptoethanol. Store in dark for <1 week.

(f) *FB_1 and FB_2 standards.*—Division of Food Science and Technology, CSIR, PO Box 395, Pretoria 00001, South Africa.

(g) *FB_1 and FB_2 standard solutions.*—Dissolve 1 mg FB_1 in 10 mL acetonitrile–water (1 + 1). Dissolve 1 mg FB_2 in 10 mL acetonitrile–water (1 + 1).

(h) *Mixed standard solution.*—Transfer 1 mL FB_1 standard solution and 500 μL FB_2 standard solution to vial; add 8.5 mL acetonitrile–water (1 + 1) to obtain mixed standard solution containing 10 ng FB_1 and 5 ng FB_2/μL.

Table 1. Recovery of fumonisins B_1 and B_2 added to corn

FB_1, μg/g				FB_2, μg/g			
Added	Total found	Net found[a]	FB_1 rec., %	Added	Total found	Net found[a]	FB_2 rec., %
0.0	0.23	0.00	—	0.0	0.05	0.00	—
0.0	0.23	0.00	—	0.0	0.07	0.00	—
0.5	0.55	0.32	64.0	0.25	0.23	0.17	67.1
0.5	0.59	0.36	71.5	0.25	0.24	0.18	72.9
1.0	0.86	0.63	63.2	0.5	0.42	0.36	72.6
1.0	0.87	0.64	64.4	0.5	0.42	0.36	72.6
2.0	1.58	1.35	67.4	1.0	0.76	0.70	70.0
2.0	1.66	1.43	71.5	1.0	0.79	0.73	73.3

[a] Corrected for control.

Table 2. Determination of fumonisins B_1 and B_2 in corn and corn screenings

Commodity	FB_1, µg/g	FB_2, µg/g	Date received	Commodity obtained from
White corn	0.14	0.05	7/90	New Orleans; FDA[a]
Corn	0.08[b]	0.03[b]	11/90	Canada; Health Protection Branch[c]
Corn LA1989	16.31[b]	4.02[b]	7/90	New Orleans; FDA
Corn MD1986	1.08	0.78	4/86	Maryland; feed store
Corn MD1987	1.04	0.31	5/87	Maryland; feed store
Corn NTRC770	0.16	0.04	3/91	New Orleans; FDA
Corn NTRC346	1.37	0.45	3/91	New Orleans; FDA
Corn screenings	106.00[b]	39.00[b]	10/90	Iowa; USDA[a]
Corn screenings	196.46[b]	42.84[b]	10/90	Iowa; USDA
Corn screenings	9.31	2.58	11/90	New Orleans; FDA
Corn screenings	4.50	1.27	11/90	New Orleans; FDA
Corn screenings	14.36	4.17	11/90	New Orleans; FDA
Corn screenings	1.74	0.59	11/90	New Orleans; FDA

[a] FDA = U.S. Food and Drug Administration; USDA = United States Department of Agriculture.
[b] Identity confirmed by mass spectrometric procedure described by Gay et al. (7).
[c] Department of Health and Welfare, Ottawa, ON, Canada K1A 0L2.

Extraction and SAX Column Chromatography

Blend 25 g ground corn with 50 mL methanol–water (3 + 1) at high speed for 2 min. Filter mixture through fluted paper and collect 10 mL extract in graduated cylinder.

Condition SAX column with 2.5 mL (1 column volume) methanol–water (3 + 1). Maintain flow rate of ca 2 mL/min and do not allow column to run dry. Add 10 mL extract to column and wash column with 2.5 mL methanol. Change receiver to 16 mL vial, and elute FB_1 and FB_2 with 14 mL methanol–acetic acid (199 + 1).

Evaporate eluate to dryness under stream of nitrogen in heating module set at 80°C. Dissolve residue in 200 µL methanol to give test solution.

Derivatization and LC Determination

Place 50 µL mixed standard solution or test solution into bottom of 2 mL vial, add 200 µL OPA reagent, mix, and immediately inject 10 µL into liquid chromatograph. Use the following settings: excitation, 335 nm; emission, 440 nm; range, 1; flow rate, 1 mL/min. FB_1 elutes as a sharp peak in 4.9 min; FB_2 elutes in 10.9 min (Figure 1).

Determine concentrations of FB_1 and FB_2 in test solution by comparing areas of chromatographic peaks obtained for test solution and standard solution. If peak areas obtained for test solution exceed those obtained for standard solution, dilute test solution and reinject.

Calculation

Calculate concentration of FB_1 and FB_2 in corn or corn product (test sample) as follows:

$$\text{ng } FB_1 \text{ or } FB_2\text{/g test sample} = (ABC)/(DE)$$

where A = peak height or area of FB_1 or FB_2 peak from test solution, B = concentration of FB_1 or FB_2 (ng/µL) in standard solution injected (i.e., 10 ng FB_1/µL or 5 ng FB_2/µL), C = final volume of test solution (typically 200 µL), D = peak height or area of FB_1 or FB_2 peak from standard solution, and E = weight of test sample represented by test solution concentration (typically 5 g). To convert ng/g to µg/g, divide by 1000.

Results and Discussion

Naturally occurring levels of FB_1 and FB_2 in control corn analyzed by the described method were found to be 0.23 and 0.06 µg/g, respectively. FB_1 and FB_2 standards were added to this corn to obtain test samples spiked at 3 levels. Duplicate test portions of these spiked test samples were analyzed, and percent recoveries were calculated after the values obtained for the control were subtracted from the levels found. The results are shown in Table 1.

We changed the original method (6) by eliminating unnecessary steps, including centrifugation after extraction, methanol-conditioning of the SAX column, and methanol–water wash of the SAX column. The mobile phase was changed from methanol–water with phosphate buffer to acetonitrile–water–acetic acid.

In this study, the average recoveries from all spiked test samples were 67% for FB_1 and 71% for FB_2. The relative standard deviations were 5% for FB_1 and 3% for FB_2. The method is rapid and easily performed. The original method (6) reported 99.5% recovery for FB_1 and 85.9% for FB_2 when standards were added to the corn extract, but no recoveries were given for the entire procedure. The estimated limit of quantitation for each metabolite is 10 ng/g. We define the limit of quantitation as the level at which the peak height is measurable above the background noise. In this study, the signal-to-noise ratios are 5:1 for FB_1 and 2:1 for FB_2 at the limit of quantitation.

Results from analyses of field corn and corn screenings are shown in Table 2 (7), and the laboratories that provided the commodities are listed. The test samples of yellow field corn

Table 3. Determination of fumonisins B_1 and B_2 in corn-based food products purchased from Washington, DC, supermarkets

Food product	FB_1, μg/g	FB_2, μg/g	Date purchased
Corn meal A	0.70	0.24	7/90
Corn meal B	1.04	0.22	7/90
Corn meal C	0.83	0.24	7/90
Corn meal D	0.77[a]	0.23[a]	7/90
Corn meal E	0.51[a]	0.05[a]	12/90
Corn meal F	0.55[a]	0.14[a]	12/90
Corn meal G	0.81[a]	0.17[a]	12/90
Corn meal H	0.79[a]	0.22[a]	12/90
Corn meal I	2.05	0.33	3/91
Corn meal J	1.04[b]	0.36[b]	3/91
Grits A	0.27	0.11	11/90
Grits B	0.18[a]	0.00[a]	12/90
Grits C	0.14[a]	0.06[a]	3/91
Corn bran cereal 90	0.33	0.04	11/90
Corn bran cereal 91	0.15	0.01	1/91
Corn bran cereal 91–2	0.13[a]	0.02[a]	3/91
Fiber cereal	0.13	0.00	1/91
Corn pops cereal	0.00	0.00	11/90
Corn cereal 90	0.00	0.00	11/90
Corn cereal 91	0.00	0.00	1/91
Corn flakes A	0.01	0.00	11/90
Corn flakes B	0.01	0.00	11/90
Corn cereal C	0.00	0.00	11/90
Corn flakes D	0.00	0.00	1/91
Corn flakes E	0.00	0.00	2/91
Tortilla chips A	0.00	0.00	3/91
Tortilla chips B	0.03	0.00	11/90
Corn chips A	0.00	0.00	11/90
Corn chips B	0.00	0.00	11/90
Corn muffin mix A	0.00	0.00	11/90
Corn muffin mix B	0.08	0.01	12/90
Tortillas A	0.00	0.00	11/90
Tortillas B	0.12	0.03	11/90
Popcorn A	0.06	0.00	11/90
Popcorn B	0.01	0.00	4/91
Hominy corn	0.06[a]	0.02[a]	4/91

[a] Identity confirmed by mass spectrometric procedure described by Gay et al. (7).
[b] Identity confirmed by mass spectrometry.

that were analyzed contained 0.08–16.31 μg FB_1/g, and the corn screenings contained 1.74–196 μg FB_1/g. The levels of FB_2 were generally around one-third of the FB_1 levels for all commodities analyzed. Table 3 shows the results of analyses of some corn-based, human food products, including corn meal and breakfast cereals purchased at local Washington, DC, supermarkets. Compared with corn, some cereals, such as corn flakes, absorbed more solvent and, therefore, were blended with 100 mL solvent rather than the 50 mL called for in the method. About half of the food products in Table 3 contained both FB_1 and FB_2. Corn meal contained the most with an average level of 0.91 μg FB_1/g. The grits and corn bran cereals both averaged 0.20 μg FB_1/g. The other corn-based foods contained from 0 to 0.13 μg FB_1/g.

Table 4. Determination of fumonisins B_1 and B_2 in corn-based food products purchased from supermarkets in Washington, DC, in August and October 1991 and in Venezuela in August 1991

Food product	FB_1, μg/g	FB_2, μg/g	Date purchased
Corn meal M	0.28	0.09	8/91
Corn meal N	1.88	0.53	8/91
Corn meal O	0.64	0.15	8/91
Grits D	0.19	0.05	8/91
Corn meal L (Venezuela)	0.07	0.02	8/91
Corn bran cereal 91–3	0.29	0.07	8/91
Fiber cereal B	0.06	0.03	8/91
Corn meal P	0.43	0.05	10/91
Corn meal Q	0.87	0.13	10/91
Corn meal R	0.63	0.12	10/91
Grits E	0.20	0.09	10/91
Corn bran cereal 91–4	0.06	0.00	10/91
Corn flakes F	0.00	0.00	10/91
Corn pops cereal	0.00	0.00	10/91

Table 4 shows the results of analyses of food products purchased in August and October 1991. The fumonisin levels found in the corn and corn products in Tables 2–4 are not corrected for percent lost during the procedure, so the true levels are probably higher than those stated in the tables. Recently, Sydenham et al. reported the presence of fumonisin contamination in human food (8). They found fumonisins in corn meal and corn grits at about the same levels as we report here.

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MYCOTOXINS

Co-occurrence of Cyclopiazonic Acid and Aflatoxins in Corn and Peanuts

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Fifty samples of peanuts and 45 samples of corn, collected in Georgia during 1990, were examined for the co-occurrence of cyclopiazonic acid (CPA) and aflatoxins (AF). The corn was collected from fields in Georgia, before harvest, under the mycotoxin-monitoring program of the U.S. Department of Agriculture's Agricultural Research Service. The peanuts were designated for oil production or animal feed because of visible damage to the nutmeats. CPA was determined by reversed-phase liquid chromatography (RPLC) using a linear gradient. Solvent A was methanol–water (85 + 15), and solvent B was 4 mM $ZnSO_4$ in methanol–water (85 + 15). The gradient started at 0% B and reached 100% B at 10 min. AF were determined by RPLC with postcolumn iodination. Of the 45 corn samples analyzed, 51% were contaminated with CPA (<25–2800 ng/g, av. 467 ng/g), and 87% were contaminated with AF (1–2300 ng/g, av. 252 ng/g). Of the 50 peanut samples, 90% contained CPA (<50–2900 ng/g, av. 460 ng/g), and 100% contained AF (3–22 000 ng/g, av. 1685 ng/g). The identity of CPA was confirmed in selected samples of corn and peanuts by mass spectrometry.

Cyclopiazonic acid (CPA) is a toxic indole tetramic acid (1–3). It has been shown that CPA is toxic to many kinds of animals, e.g., rats (4), dogs (5), pigs (6), and chickens (7), and mutagenic for *Salmonella typhimurium* TA 98 in the Ames test (8). CPA is produced by several species of *Aspergillus* and *Penicillium*, including *A. flavus*, *A. oryzae*, *P. cyclopium*, and *P. camemberti* (1, 2, 9, 10). Some strains of *A. flavus* produce both CPA and aflatoxins (AF) (11). A study by Gallagher et al. (12) indicated that *A. flavus* isolated from various sources produces CPA more frequently than AF; 52% of the *A. flavus* isolates produced CPA, whereas 33% produced AF. CPA has been found as a contaminant in corn (11), peanuts (13), and millet implicated in human disease in India (14), and as a residue in poultry meat from animals dosed orally with CPA (15). AF have also been found in these food commodities. Both CPA and AF have been shown to cause health problems in animals and humans. However, only limited surveys have been performed to monitor CPA in agricultural commodities (16). This paper reports the co-occurrence of CPA and AF in corn and peanuts, as found in a survey conducted as part of the mycotoxin-monitoring program of the U.S. Department of Agriculture's Agricultural Research Service (ARS).

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Sampling

In August 1990, 45 samples of corn were collected from corn fields before harvest. The fields were located within a 100 mile radius of Tifton, GA, where drought was severe from July to November 1990. The corn was shucked and shelled. Each laboratory sample of shelled corn weighed 3–5 lb. Fifty samples of peanuts were taken from stock peanuts that had been collected by ARS because of the severe and prolonged late-season drought in 1990. The peanut samples were taken from farmers' trailers, which had been brought directly to the buying point from the field. Because the nutmeats had visible damage by *A. flavus* and *A. parasiticus*, the peanuts had been designated for use in oil production or for animal feed. Each laboratory sample of shelled peanuts weighed 5–15 lb.

Experimental

AF were extracted from corn and peanuts with methanol–water (8 + 2). Corn extracts were cleaned up on a Sep-Pak silica gel cartridge (17), and peanut extracts were purified on a Florisil–alumina minicolumn (18). AF were determined by reversed-phase liquid chromatography (RPLC) with postcolumn iodination (18). The mobile phase was water–tetrahydrofuran (8 + 2) at a flow rate of 1.5 mL/min. The flow rate for the saturated aqueous iodine solution was 0.4 mL/min.

CPA was determined, and its identity was confirmed by a previously reported method (19). CPA was extracted from corn and peanuts with methanol–2% sodium bicarbonate solution

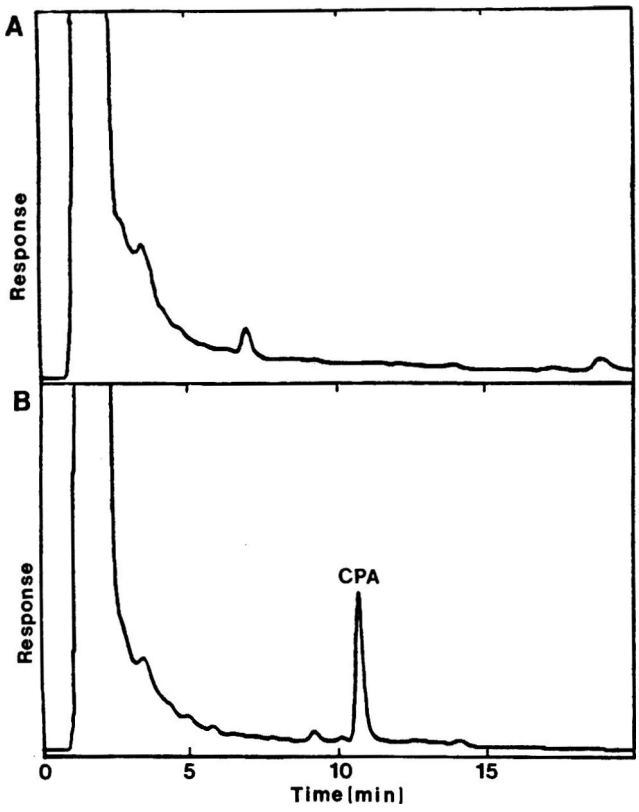

Figure 1. Liquid chromatograms of (A) control corn and (B) naturally contaminated corn (CPA at 230 ng/g).

(7 + 3) and partitioned into chloroform. The chloroform extract was passed through a Sep-Pak silica gel cartridge, and CPA was determined by RPLC with a 10 min linear gradient. Solvent A was methanol–water (85 + 15), and solvent B was 4mM $ZnSO_4$ in methanol–water (85 + 15). The gradient started at 0% B and reached 100% B in 10 min. The flow rate was 1.0 mL/min. The fraction presumed to contain CPA was collected from the LC column, the eluate was evaporated, and the residue was dissolved in chloroform; 2 µL of the solution was introduced into the mass spectrometer, and a spectrum was obtained.

Results and Discussion

CPA and AF were determined in 45 samples of corn and in 50 samples of peanuts that had been collected in Georgia during 1990. Figure 1 shows a typical liquid chromatogram of control corn and naturally contaminated corn. The LC fraction presumed to contain CPA was collected and examined by positive-ion chemical ionization (PICI) tandem mass spectrometry (MS/MS) (19). The identity of CPA was confirmed by comparison of the mass spectrum of the presumed CPA with the mass spectrum of the CPA standard solution. The spectra were identical (Figure 2).

Because all the liquid chromatograms for contaminated corn and peanuts were similar, PICI MS/MS was used to confirm the identity of CPA in only 2 test samples each of corn and peanuts.

Results of the analyses of corn and peanuts are summarized in Figures 3 and 4, respectively. Of the corn test samples, 51% contained CPA (<25–2800 ng/g, av. 467 ng/g), and 87% contained AF (1–2300 ng/g, av. 252 ng/g). Of the peanut test samples, 90% contained CPA (<50–2900 ng/g, av. 460 ng/g), and 100% contained AF (3–22 000 ng/g, av. 1685 ng/g). CPA and AF were found in the same test samples for 51% of the corn and 90% of the peanuts; however, 36% of the corn and 10% of the peanut test samples contained only AF, and 13% of the corn test samples contained no CPA or AF (Tables 1 and 2).

Differences in limits of determination might explain the discrepancies in the number of test samples contaminated by each

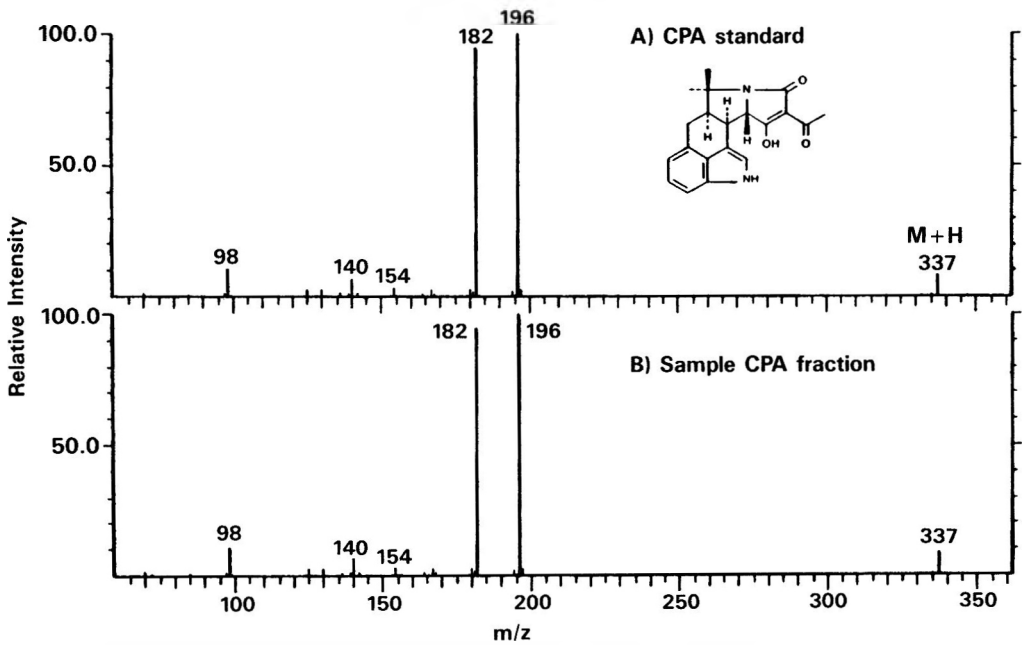

Figure 2. PICI MS/MS spectra of (A) CPA standard solution and (B) LC fraction containing CPA from naturally contaminated corn (820 ng/g).

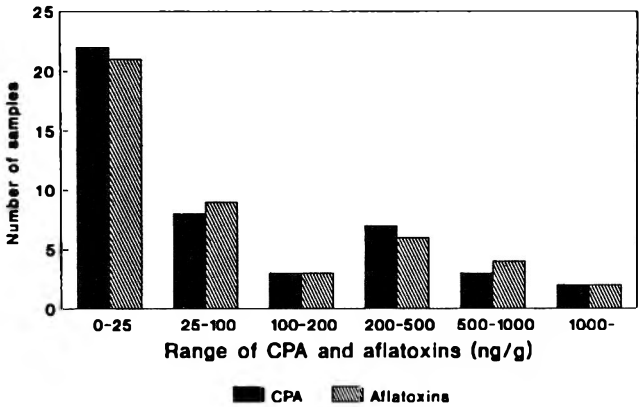

Figure 3. CPA and AF in corn. The limit of determination for CPA in corn was 25 ng/g.

analyte. The limit of determination for AF was 1 ng/g for both commodities, whereas the limits of determination for CPA were 25 ng/g for corn and 50 ng/g for peanuts. We define the limit of determination as the level at which the peak height is measurable above the background noise. In this study, the signal-to-noise ratio was 5:1.

The levels of CPA contamination were found to be similar for the 2 commodities, whereas AF levels in the peanuts were about 10 times those in the corn. The peanuts were selected from stocks that were visibly damaged, which might explain the higher AF contamination levels in those samples.

Other explanations for the higher incidence and levels of AF found in peanuts could be that the peanuts were colonized by *A. parasiticus* that did not produce CPA or by *A. flavus* that produced CPA at levels too low to be determined. When the CPA level was high, the AF level was high also; a similar relationship usually existed for low levels.

Another result noted in this study was that 58% of the peanuts were contaminated with the aflatoxin G group as well as with the aflatoxin B group, whereas 75% of the corn was found to contain only the aflatoxin B group. It appears that most of the corn was colonized mainly by *A. flavus*, which produces only the aflatoxin B group (20), and that many of the peanuts were colonized not only by *A. flavus* but also by *A. parasiticus*,

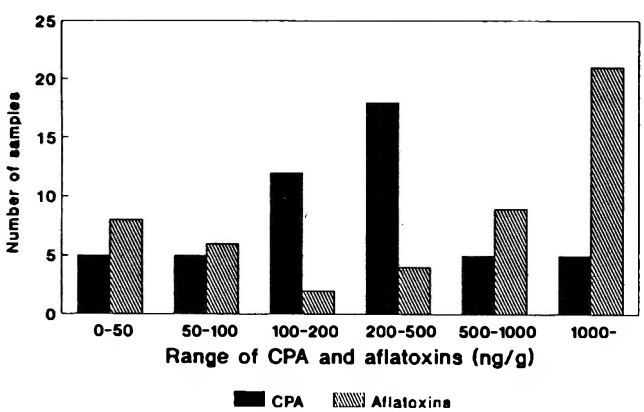

Figure 4. CPA and AF in peanuts. The limit of determination for CPA in peanuts was 50 ng/g.

Table 1. Concentration of cyclopiazonic acid (CPA) and aflatoxins (AF) in corn

		AF, ppb				
Lot No.	CPA, ppb	Total	B_1	G_1	B_2	G_2
1	T[a]	128	121	ND[b]	7	ND
2	250	256	245	ND	11	ND
3	174	166	161	ND	5	ND
4	114	204	187	9	9	ND
5	ND	6	5	ND	1	ND
6	220	250	239	ND	10	ND
7	480	373	356	ND	17	ND
8	797	749	735	ND	14	ND
9	T	51	50	ND	1	ND
10	ND	6	6	ND	T	ND
11	120	797	760	ND	37	ND
12	770	527	515	ND	12	ND
13	ND	ND	ND	ND	ND	ND
14	ND	ND	ND	ND	ND	ND
15	89	58	56	ND	2	ND
16	ND	ND	ND	ND	ND	ND
17	T	14	14	ND	T	ND
18	ND	16	16	ND	T	ND
19	1598	2280	2230	ND	51	ND
20	ND	1	1	ND	ND	ND
21	633	354	346	ND	8	ND
22	ND	15	15	ND	T	ND
23	ND	6	6	ND	ND	ND
24	ND	5	5	ND	ND	ND
25	225	143	138	ND	5	ND
26	ND	ND	ND	ND	ND	ND
27	ND	11	10	1	T	ND
28	ND	35	34	ND	1	ND
29	2771	1645	1600	ND	45	ND
30	231	265	242	8	16	ND
31	ND	42	38	ND	4	ND
32	ND	98	53	36	5	5
33	ND	12	12	ND	T	ND
34	47	21	21	ND	T	ND
35	ND	ND	ND	ND	ND	ND
36	ND	ND	ND	ND	ND	ND
37	29	54	52	ND	2	ND
38	ND	21	20	ND	1	ND
39	ND	10	10	ND	ND	ND
40	ND	23	23	ND	T	ND
41	49	10	10	ND	ND	ND
42	358	318	306	ND	12	ND
43	322	672	654	ND	18	ND
44	ND	57	55	ND	2	ND
45	53	98	82	13	3	T

[a] Trace; below the limit of determination.
[b] None detected.

which produces both the aflatoxin B and aflatoxin G groups (20).

This study provides evidence that corn and peanuts can be contaminated simultaneously with CPA and AF. The data from

Table 2. Concentration of cyclopiazonic acid (CPA) and aflatoxins (AF) in peanuts

Lot No.	CPA, ppb	AF, ppb				
		Total	B_1	G_1	B_2	G_2
1	523	1245	1131	ND[a]	114	ND
2	275	1907	1605	154	138	10
3	161	925	750	124	46	5
4	234	1372	1152	116	96	8
5	1223	3187	2522	399	225	41
6	873	3351	2616	458	233	44
7	731	2903	2482	167	235	19
8	252	128	121	ND	7	ND
9	132	48	42	3	3	ND
10	198	1170	1018	37	115	ND
11	216	1058	895	75	87	ND
12	73	390	323	38	29	ND
13	1262	2396	2215	ND	181	ND
14	919	3949	3413	264	253	20
15	276	1303	1121	97	85	ND
16	207	687	643	ND	44	ND
17	227	1042	915	60	67	ND
18	306	833	780	ND	53	ND
19	ND	23	18	2	3	ND
20	ND	32	20	5	5	1
21	158	58	47	8	4	ND
22	432	67	61	ND	6	ND
23	62	38	33	ND	5	ND
24	244	57	53	ND	4	ND
25	191	39	38	ND	2	ND
26	258	1189	1020	79	91	ND
27	265	2114	1824	132	147	11
28	195	1258	960	216	70	12
29	2926	22186	17203	2331	2245	407
30	2254	18203	14017	1911	1883	392
31	T[b]	567	390	101	54	22
32	145	695	495	113	60	28
33	192	197	185	ND	12	ND
34	397	258	231	12	16	ND
35	203	1116	1012	ND	104	ND
36	164	1467	1335	ND	132	ND
37	283	919	863	ND	56	ND
38	165	681	621	ND	60	ND
39	172	7	5	1	1	ND
40	ND	355	318	ND	37	ND
41	ND	56	38	8	9	1
42	ND	3	2	ND	1	ND
43	51	54	40	2	12	ND
44	88	49	40	2	7	ND
45	235	97	48	32	15	2
46	446	800	754	ND	46	ND
47	406	1318	1240	ND	78	ND
48	469	367	324	ND	43	ND
49	645	1101	1019	ND	82	ND
50	1106	902	838	ND	64	ND

[a] None detected.
[b] Trace; below the limit of determination.

this study support the contention that both CPA and AF are produced by *Aspergillus flavus*.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Characterization of the O-(2,3,4,5,6-Pentafluorobenzyl)-hydroxylamine Hydrochloride (PFBOA) Derivatives of Some Aliphatic Mono- and Dialdehydes and Quantitative Water Analysis of These Aldehydes

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A study was conducted to validate the use of O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBOA) as an agent for the determination of formaldehyde, acetaldehyde, *n*-heptanal, *n*-decanal, and glyoxal, aldehydes commonly found as by-products in drinking and other water after a prior ozonation step. The study involved synthesis and characterization of the pure PFBOA-oxime derivatives and their subsequent use in validation studies in various types of chemically defined waters. The yields of the synthesized PFBOA-oximes exceeded 80%. They were characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, ultraviolet (UV) and infrared (IR) spectrophotometry, and gas chromatography/electron impact positive ion mass spectrometry (GC/MS) to assess their purity and define the suitability of these techniques in aldehyde quantitation. The *syn/anti* isomers of the oximes were detected by GC/MS and both NMR techniques, but not by IR or UV. The most sensitive technique was selected ion monitoring GC/MS, which allowed picogram quantitation over a wide linear range. A high PFBOA concentration (10 mg/mL) with 3 hexane extractions, initial pH adjustment, and microwave heating proved superior (>80% recoveries) to a published method involving room temperature, low PFBOA concentration (1 mg/mL), and a single hexane extraction for many defined waters. The use of standard reference materials as defined matrixes for aldehyde spiking recovery experiments to account for matrix effects is novel, and demonstrates that the method is quantitative (>80% recoveries) in a wide variety of matrixes.

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Environmental analytical chemists are continually developing and improving methods to detect toxic contaminants in all environmental media. Often, methods must be developed to enforce regulations, such as those of the U.S. Environmental Protection Agency to set maximum contaminant levels of by-products from the use of treatment chemicals for the production of drinking water (1).

To ensure that a particular method provides accurate and precise results independent of sample matrix, authentic standards are essential (2). O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBOA) has been shown to be a promising derivatizing agent for the detection of lower molecular weight aliphatic mono- and dialdehydes, which are by-products in ozonated water (3–6). The corresponding aldehyde oximes are easily resolved by gas chromatography (GC) and are amenable to detection by electron capture detection (ECD) techniques (7–9). There are few data on the physical and spectroscopic characteristics of these PFBOA–aldehyde oxime derivatives, because they are not commercially available.

Most previous studies using PFBOA (3–9) have assumed that derivatization efficiency, extraction in hexane, and aldehyde-oxime recovery are adequately accounted for by comparison to an internal standard, decafluorobiphenyl, carried through the procedure when known amounts of aldehydes are added to distilled water at concentrations typically found in the environment (parts per billion). The procedure can only be valid if the compound chosen as internal standard is an adequate surrogate for the oxime derivatives, and if the reaction has 100% efficiency.

The only way to determine and distinguish reaction efficiency, extraction recovery, and overall accuracy is to use authentic standards. These standards must be of high purity and be used under conditions encountered in the environment as well as in the analytical method (2). Absolute response factors for mass spectrometric (MS) or ECD measurements can be determined only by use of accurately known amounts of pure standards. Once absolute recoveries can be calculated, the contributions of extraction efficiency, cleanup, reaction efficiency, and water matrix can be understood. The present paper describes this process for the reaction of PFBOA and selected

Table 1. Amounts and vessels used for the synthesis of the oximes of some aldehydes (C1, formaldehyde; C2, acetaldehyde; C7, *n*-heptanal; C10, *n*-decanal; Gly, glyoxal) with PFBOA

Starting aldehyde	Aldehyde volume, μL	Reaction vessel[a]	PFBOA		PFBOA/aldehyde, molar ratio
			Mass, mg	Water, mL	
C1	62.2[b]	16 × 150	206.5	10	1.0
C2	51.7	16 × 150	204.0	10	0.907
C7	114.3[c]	16 × 150	200.8	10	1.0
C10	143.3[d]	65 mL[e]	186.6	10	1.0
Gly	137.0[f]	25 × 200	1213.9	25	4.1

[a] Screw cap culture vessel (Kimax culture tube) with outer diameter and length given in mm, respectively.
[b] As 36.9% aqueous solution.
[c] In 2 mL methanol solution.
[d] In 15 mL methanol solution.
[e] In a pear-shaped flask.
[f] As 40% aqueous solution.

aliphatic aldehydes that are generally found when ozonation is used to produce drinking water.

Experimental

Chemicals

Formaldehyde (C1: 37% in aqueous solution) was obtained from Fisher (Pittsburgh, PA). Acetaldehyde (C2: 99%), *n*-heptanal (C7: 95%), *n*-decanal (C10: 95%), glyoxal (Gly: 40% in aqueous solution), decafluorobiphenyl (DFB), pentafluorobenzyl aldehyde (PFBA: 98%), and pentafluorobenzyl alcohol (PFBOH: 98%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). C7 and C10 were >98% pure by GC/MS. The aldehyde concentrations of the aqueous solutions were checked after filtration through a 0.45 μm Teflon filter by the sodium bisulfite–iodine titration method (10). PFBOA from Aldrich Chemical Co. or Lancaster Synthesis (Windham, NH) was used without further purification. National Institute of Standards and Technology materials were also used, including Standard Reference Material (SRM) 1643b Trace Elements in 0.5M HNO_3, SRM 2694a-I Simulated Rainwater, and SRM 2694a-II Simulated Rainwater. Los Angeles tap water was also used.

Distilled water was passed through a Millipore Super-Q water filter system to produce ASTM Type I (11) water before use. Analysis by simultaneous inductively coupled plasma atomic emission spectroscopy of the ASTM Type I water revealed the following elemental concentrations in mg/L: Al, 0.023; As, 0.002; B, 0.054; Ca, 0.19; K, 0.17; Li, 0.005; Mg, 0.11; Mo, 0.006; Si, 3.1; Sr, 0.002; and Zn, 0.001. Other elements sought but not detected were Ag, Ba, Be, Cd, Co, Cr, Cu, Fe, Mn, Na, Ni, P, Pb, Se, Ti, and V. Water used for recovery experiments had to be further treated because of the high Cl concentration [distilled from 64 mg potassium permanganate in 500 mL water plus 1 mL concentrated sulfuric acid (11)]. Hexane and methanol (Fisher, OPTIMA) were used as solvents. Deuterated chloroform ($CDCl_3$) containing 1% tetramethylsilane (TMS) and deuterated water (D_2O) containing 1% sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) originated from MSD Isotopes (St. Louis, MO). Carbon tetrachloride was obtained from Allied Chemical (Morristown, NJ). Nitrogen (99.995%) from Alphagaz Specialty Gases was passed through a charcoal filter (20/40 mesh, Aldrich Chemical Co.) prepared in a Pasteur pipet before use.

Synthesis of Oximes

All glassware was washed with detergent, rinsed with distilled water, soaked at least 24 h in a 10% nitric acid bath, rinsed in distilled water, and oven-dried at 100°C.

The PFBOA derivatives were synthesized in 150–200 mg quantities by reacting the aldehyde with the appropriate amounts of PFBOA prepared as aqueous solutions (Table 1). The PFBOA solutions were ultrasonicated (B22DOR-1, Branson Ultrasonics Corp., Danbury, CT) to aid solubility. The individual aldehydes were added slowly with shaking to the appropriate PFBOA solutions contained in the appropriate containers (Table 1). The less water-soluble C7 and C10 were added as methanol solutions to promote enough water solubility to facilitate reaction. C1 and Gly were added as aqueous solutions. C2 was added while cold as a specific volume of pure liquid calculated from the density. Equimolar amounts of PFBOA to aldehyde were used for all except C2 and Gly (Table 1). Less than excess C2 was added because of its volatility and because excess PFBOA could be removed easily by copious water washing. Excess Gly was added to ensure reaction to produce the di-substituted Gly derivative.

All solutions became turbid on aldehyde addition; the PTFE-lined screw caps were replaced, and the tubes were shaken vigorously and then heated in a microwave oven until the first bubbles appeared (generally 10–15 s for a solution temperature of ca 80°C). The solutions were cooled in an ice bath at 0°C, and except for the C10 derivative, were then centrifuged at 300 × *g* for 5 min at 5°C. The oxime appeared as a solid or liquid layer at the bottom of each tube. The upper aqueous layer was removed by Pasteur pipet except for the Gly de-

rivative. The oxime was recovered by extraction of the remaining bottom layer with three 1 mL portions of hexane. Each hexane solution in a 5 mL graduated V-vial (Kimax 49200) was evaporated under a stream of nitrogen. The removed upper aqueous layer was also extracted with three 1 mL portions of hexane, and any oxime was also recovered as above in a separate V-vial.

The Gly derivative precipitate was vacuum-filtered onto a Nylon 0.45 μm membrane while still cold and washed with two 2 mL portions of cold Type I water; the filter and contents were transferred to a watch glass when dry. The C10 derivative was synthesized in a 65 mL pear-shaped flask. C10 was added to the aqueous PFBOA in 15 mL methanol. The ground glass stopper of the flask was replaced, and the flask was shaken vigorously. After the microwave step, the excess methanol was evaporated on a heating mantle. On cooling to 0°C, excess C10 formed a waxy ring at the air/solution interface and was carefully removed by a spatula. The C10 derivative was then extracted with three 1 mL portions of hexane. All oximes were dried in a vacuum desiccator until constant weight was achieved for 2 consecutive days. The materials were then stored at −20°C.

Monosubstituted Gly and Propanal Oximes

Because Gly has 2 carbonyl groups, it is likely that the monosubstituted derivative may be produced in situations in which less than excess PFBOA is present. To synthesize the monosubstituted derivative, 90.5 mg (0.36 mmol) PFBOA was added directly to a 20 mL test tube containing 1240 mg of a 40% aqueous solution of Gly (8.6 mmol). A white precipitate formed almost immediately. After the mixture was allowed to react at room temperature for 1 h, the precipitate was collected on a 0.45 μm Nylon membrane filter by vacuum filtration. The precipitate was washed with 2 mL Type I distilled water and dried to constant weight.

From GC studies, the retention times of the PFBOA monosubstituted Gly and propanal derivatives were expected to be similar. To investigate this situation, 5 mL of an aqueous solution containing 0.0089 mmol Gly was added to 5 mL of an aqueous solution containing 0.0065 mmol propanal, and 2 mL of an aqueous solution containing 0.009 mmol PFBOA was added. The mixture was allowed to react at room temperature for 1 h, shaken for 30 s, and then extracted with 1 mL hexane.

To illustrate that the mono-Gly PFBOA-oxime GC peak disappeared when excess PFBOA was present, 5 mL of an aqueous solution containing 0.0089 mmol Gly was added to 5 mL of an aqueous solution containing 0.0065 mmol propanal, and 10 mL of an aqueous solution containing 0.045 mmol PFBOA was added. The mixture was extracted as above.

GC/MS Analysis

MS data were obtained on a Hewlett-Packard 5970B mass selective detector operating in the full scan mode for characterization (50–450 atomic mass units) or in selected ion mode (SIM) for purity determinations (ions 181, 196, 198, and 334). MS ions of *m/z* 181, 196, 198, and 334 were the base peaks of the PFBOA oxime derivative and PFBOA, PFBA, PFBOH, and the DFB internal standard, respectively. Positive ions were produced in a 70 eV source at 300°C. GC was conducted on a Hewlett-Packard 5890 gas chromatograph equipped with a DB-5 chemically bonded fused-silica capillary column, 30 m × 0.25 mm id (J & W Scientific). Samples were injected in the splitless mode, with a purge delay of 0.75 min and an injection port temperature of 250°C. Helium was used as the carrier gas at a flow rate of 3 mL/min. The temperature program was as follows: 50°C for 1 min, 50°C to 250°C at 5°C/min, holding at 250°C for 4 min. The purities of oximes corrected for the presence of PFBOH and PFBA were obtained by the method of external standards in the linear response region (20–1000 ppb) for an injection volume of 4 μL. The apparent purities of the oxime derivatives were computed from percentage areas from GC/MS, assuming equal response factors for all eluted compounds. Corrected purities were calculated from percentage areas from GC/MS-SIM, using calibration curves for the key ions of known impurities.

GC/MS response factors and linear dynamic ranges were determined by triplicate 4 μL injections (sandwich technique) of solutions generated by stepwise dilution of the pure oxime derivatives with hexane, starting with concentrations of 0.575, 0.295, 0.350, 0.387, and 0.185 mg PFBOA oxime/mL hexane for C1, C2, C7, C10, and Gly PFBOA oxime derivatives, respectively. To 1 mL of each diluted solution, 10 μL of a 40 μg/mL hexane solution of DFB was added as internal standard. The total area for the *anti* and *syn* isomers for *m/z* 181 relative to the area for the internal standard for *m/z* 334 was calculated for each injected mass. The PFBOA oxime concentrations generated were between 0.287 and 875 ng PFBOA oxime/mL hexane in addition to the control. At least 9 different concentrations in triplicate were used to define the GC/MS linear range and response factors. Linear regression allowed the response factor to be calculated from the slope of the linear region, the latter being defined from the injected mass range that allowed a precision of ±10% and accuracy of at least 90%. Detection limits for each PFBOA oxime were calculated by using the criterion of 3.29 times the control signal (12). Intrasample and intersample precisions for same and different day injections were defined for the PFBOA oximes and DFB.

NMR Spectral Analysis

All ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM360/WB Fourier transform spectrometer equipped with a 8.5 Tesla superconducting magnet. Spectra were recorded at ambient temperature, using a spectral width of 10 000 Hz for ^{1}H and 25 000 Hz for ^{13}C. All ^{13}C spectra were broadband decoupled at a decoupling power of 15 Hz, with a relaxation delay of 20 s. Proton spectra were acquired by using a 5 mm sample tube (Aldrich Chemical Co.); ^{13}C spectra were measured by using a 10 mm sample tube (Aldrich Chemical Co.). Oxime samples were dissolved in $CDCl_3$ containing 1% tetramethylsilane (TMS). The spectra of PFBOH in $CDCl_3$ and PFBOA in D_2O were also measured to aid in peak assignments. All spectra were corrected for the blank. The reference compound for D_2O solutions was DSS.

IR Spectral Analysis

IR spectra were obtained on a Perkin-Elmer model 1600 Fourier transform single beam IR spectrophotometer in 1 mm sodium chloride liquid cells. Samples were prepared in carbon tetrachloride and scanned from 500 to 3000 cm^{-1} at 4 cm^{-1} resolution. The spectra were corrected for the blank.

UV Spectral Analysis

UV spectra were obtained between 190 and 820 nm on a Hewlett-Packard 8451A diode array single beam spectrophotometer of 2 nm bandwidth, using a 1 cm Suprasil cell. Molar absorptivities were determined at 222 and 264 nm by linear regression of the Beer's law region found by progressive dilution in acetonitrile. The spectra were corrected for the solvent. The spectrum of PFBOA was measured in Type II water.

Analysis of Aldehydes in Water

The optimized method was compared with a published method (4) in which 2 drops of 0.1M sodium thiosulfate solution (to reduce hypochlorite in chlorinated waters) and then 0.5 mL of a 1 mg/mL aqueous PFBOA solution were added to 5 mL portions of water in 10 mL screw-capped culture tubes. The solution was allowed to stand at room temperature for 2 h and then acidified with 1 drop of 18N sulfuric acid (to hydrolyze excess PFBOA). Oxime derivatives were extracted in the same vial with 1 mL hexane containing DFB by shaking for 30 s. The upper hexane layer was transferred by Pasteur pipet to another vial, shaken with 5 mL 0.1N sulfuric acid (to completely remove PFBOA, which interferes at high mass with the C2 derivative), transferred again, and dried by adding about 50 mg anhydrous sodium sulfate. GC/ECD or GC/MS analysis then followed.

The optimized method also began with a 5 mL water sample. A 0.100 mL aliquot of 0.1N sodium thiosulfate was added with shaking, enough 6N HCl or 6N NaOH was added to adjust the pH to 5–6 (this step was omitted if the pH was between 1 and 7), and 0.5 mL of a 10 mg/mL solution of PFBOA was added, plus enough HCl to adjust the final pH to 0.9–1.3 (a total of 25 μL HCl for distilled water samples, for example). After shaking, the samples were microwaved for 30 s to a temperature of about 80°C and then cooled to room temperature, and finally 50 μL 18N sulfuric acid was added with shaking. The solutions were extracted with three 1 mL portions of hexane containing DFB; the first extraction contained the internal standard. All extracts were then combined in a 4 mL vial, ca 50 mg anhydrous sodium sulfate was added, and the sample was shaken. A 4 μL injection of the hexane fraction was then analyzed by GC/MS.

Various types of waters spiked with aldehydes were evaluated by both methods in triplicate, including ASTM Type I water that had been distilled from acid permanganate, SRM 1643b Trace Elements in Water, SRM 2694a-I Simulated Rainwater, SRM 2694a-II Simulated Rainwater, and Los Angeles tap water from the distribution system. The aldehyde concentrations in ng/mL were as follows: C1, 333; C2, 202; C7, 518; C10, 691; and Gly, 68. These high concentrations were necessary to be clearly in the linear dynamic range. All determinations were corrected for the blank.

Results and Discussion

Characterization of PFBOA Derivatives

Data for the PFBOA derivatives are classified in all tables as a function of their starting aldehyde: C1 (formaldehyde), C2 (acetaldehyde), C7 (*n*-heptanal), C10 (*n*-decanal), and Gly. All derivatives, except C1, exist as *anti* (I) and *syn* (II) isomers ($C_7H_2F_5O$ is *anti* or *syn* to the proton equivalent to the *Z* and *E* diastereomers of the Cahn-Ingold-Prelog system of nomenclature, respectively), with the *bis* oxime of Gly having 3 possible geometric isomers, *syn/syn* (III), *syn/anti* (IV), and *anti/anti* (V) (Figure 1). No attempt was made to isolate pure isomers. The total area for both isomers was used to quantitate each PFBOA derivative.

MS data for the PFBOA derivatives are summarized in Table 2. The base peak for all the derivatives is *m/z* 181, the pentafluorotropylium cation (4). This common base peak allows SIM for all the aldehyde PFBOA derivatives. Molecular ions were observed in the C1, C2, and Gly derivatives but not in the C7 or C10. Other common ions were *m/z* 167 (C_6F_5+), *m/z* 155 (C_5F_5+), *m/z* 93 (C_3F_3+), and *m/z* 62 (C_2F_2+), all between 1 and 5% of the base peak. C7 and C10 derivatives show a typical hydrocarbon pattern with successive loss of *m/z* 14 at the lower mass ranges. GC conditions allowed the various *syn/anti* isomers to be separated. Only 2 Gly isomers were resolvable, not the expected 3 isomers. No differences could be seen in the mass spectra of isomers.

Table 3 shows the apparent GC purity, assuming equal response factors, and the corrected purity accounting for the response factors of PFBOH and PFBA. Exact MS mass measurements verified the expected elemental composition of those derivatives having molecular ions.

The MS response factors were obtained from the slopes of the linear regression of the area units for *m/z* 181 divided by the area units for *m/z* 334 by multiple SIM of *m/z* 181, 196 (PFBA), 198 (PFBOH), and 334 (DFB) that corresponded to the picomoles of PFBOA–aldehyde derivative injected in the linear dynamic range. All correlation coefficients were >0.98. All intercepts were indistinguishable from 0. The MS response values in normalized area units/pmol of injected PFBOA derivative were as follows: C1, 0.251; C2, 0.236; C7, 0.165; C10, 0.0979; and Gly, 0.187. The respective percent relative errors were 0.015, 0.33, 0.013, 0.013, and 0.052. As the chain length became longer for the normal-chain derivatives, the MS response factor decreased linearly according to the following equation:

$$\text{response factor} = -0.0165 \times \text{number of carbon atoms in the normal-chain aldehyde} + 0.2701$$

with $r = -0.994$. Thus, the MS response factors for any normal-chain aldehyde derivative of PFBOA between C1 and C10 can be calculated. Adding *m/z* 181 ion current values will not allow

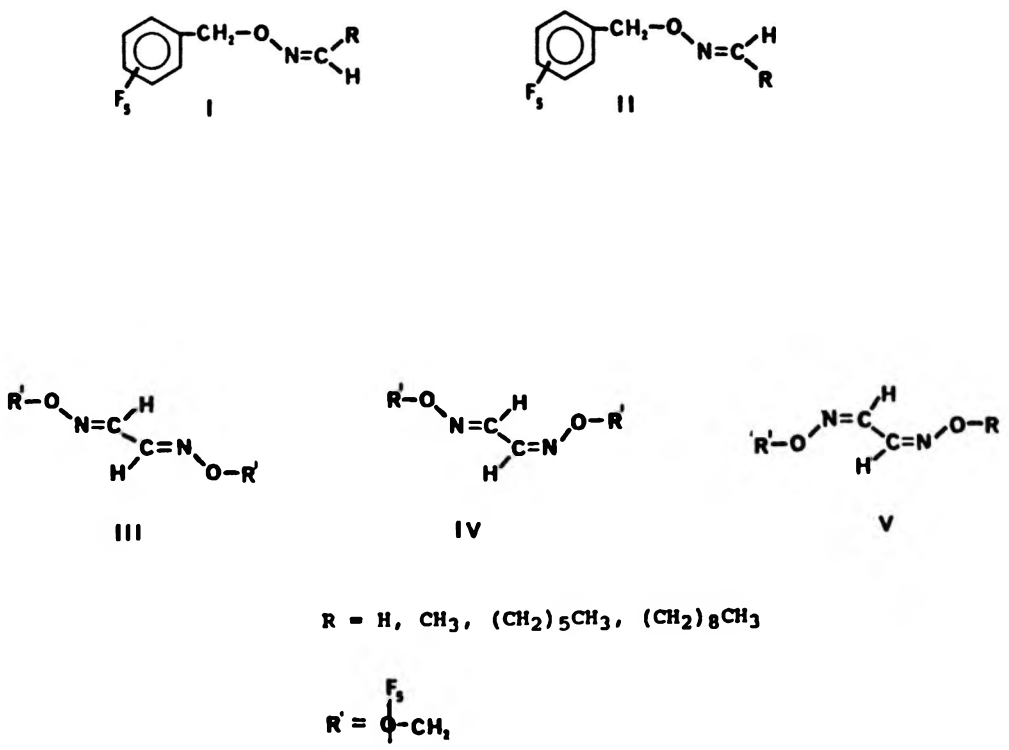


Figure 1. ***Anti*** **(I) and** ***syn*** **(II) forms of long-chain PFBOA-oximes;** ***syn/syn*** **(III),** ***syn/anti*** **(IV), and** ***anti/anti*** **(V) forms of PFBOA–glyoxal oxime.**

calculation of the total aldehydes present unless they are weighted by their response factors before the addition.

The response of the Gly PFBOA compound, as expected for a disubstituted derivative, does not fit the normal chain monoaldehyde relationship, its response factor being equivalent to that of the *n*-pentanal PFBOA derivative. The respective observed linear ranges/detection limits (pg) were C1, 23–2300/2.3–23; C2, 240–1200/120–240; C7, 360–3500/140–360; C10, 310–1600/160–310; and Gly, 300–1500/70–300. Most lower limits of the observed linear range were about 250 pg, except C1, for which the limit was 23 pg because C1 had no *anti-* or *syn-* isomers and, because it had the shortest retention time, the peak was sharp. When the concentration factor of 5 mL water sample/3 mL hexane extraction volume for the optimized method was factored in, the lowest water concentration measured accurately to within 10% precision was about 40 ng/mL, except for C1, for which it was 14 ng/mL. The detection limits were about 170 pg for aldehydes except C1, for which the detection limit was about 10 pg. The corresponding detection limits for water concentrations were about 26 and 1.5 ng/mL, respectively.

To obtain quantitative results in further method development, spiking had to occur in the linear dynamic range. Increased sensitivities were obtained by decreasing the combined extraction volume of hexane with a stream of nitrogen; the control sample was treated similarly. Decreasing the hexane volume used for each individual extraction and increasing the water volume assayed would require revalidation of the method. The intrasample precision for the PFBOA oximes and DFB was <4%, and the intersample precision on the same day was <5%. The inter- and intrasample precisions on different days varied between 3 and 15%. Therefore, all comparative analyses were performed on the same day.

^{1}H NMR spectra (Table 4) further demonstrate the existence of *syn/anti* isomers for the monosubstituted PFBOA derivatives beyond C1. The *syn/anti* ratio was 1.71 ± 0.10 (arithmetic mean ± standard deviation) for the normal-chain aldehyde PFBOA-oximes on the basis of evidence from 8 sets of data. This ratio varied for hours immediately after synthesis. All the NMR data were obtained for compounds stored at least overnight at –20°C before NMR analysis; beyond this storage time, the spectra did not change. The *syn* form, thus, comprises about 63 ± 4% of the PFBOA-oxime. Three distinct signals provided *syn/anti* information: the proton on the oxime carbon (*syn* 7.35–7.40 ppm, *anti* 6.66–6.78 ppm), the –CH_2–O protons (*syn* 5.09–5.10 ppm, *anti* 5.15–5.17 ppm), and the –CH_2– alpha to the oxime carbon (*syn* 2.14–2.15 ppm, *anti* 2.27–2.28 ppm). Other investigators have shown the *syn* forms of the oxime O-methyl ethers to be more stable than the *anti* form (13, 14). The proton NMR data for the PFBOA–Gly derivative show protons alpha to the aromatic ring at *syn* 5.23 ppm and *anti* 5.24 ppm. The proton of the carbonyl carbon had 3 distinct signals tentatively assigned as *syn/syn* 8.16 ppm (doublet, J = 8.84 Hz), *anti/anti* 7.13 ppm (doublet, J = 8.69 Hz), and *syn/anti* 7.68 ppm (singlet). The hydrogens on the oxime carbon for the C1 derivative are not magnetically equivalent, and each split each other's levels to form 2 doublets.

The ^{13}C NMR spectra further demonstrate the existence of *syn/anti* isomers (Tables 5 and 6). Two signals are observed for the oxime carbon in all normal-chain derivatives apart from C1

Table 2. Mass spectra of PFBOA-oxime derivatives of formaldehyde (C1), acetaldehyde (C2), *n*-heptanal (C7), *n*-decanal (C10), and glyoxal (Gly)

Starting aldehyde	MW[a]	Major ions (relative intensity)													
C1	225	181	(100)	195	(11)	161	(10)	117	(9)	182	(7)	99	(7)	167	(5)	93	(5)
C2	239	181	(100)	209	(8)	182	(7)	161	(6)	117	(5)	195	(6)	167	(4)	99	(4)
C7	309	181	(100)	239	(29)	55	(9)	182	(4)	207	(6)	128	(5)	69	(5)	161	(4)
C10	351	181	(100)	239	(31)	55	(14)	57	(10)	69	(9)	182	(7)	170	(6)	207	(5)
Gly	448	181	(100)	448	(12)	182	(7)	161	(5)	167	(4)	117	(4)	99	(4)	—	—

[a] MW = mass spectral molecular weight.

(151 to 157 ppm, *syn*; 151 to 156 ppm, *anti*; Table 6) as well as for the methylene carbon alpha to the aromatic ring (65.4 to 67.9 ppm, *syn*; 65.0 to 66.7 ppm, *anti*; Table 5). Carbon atoms alpha to the oxime carbon also show *syn/anti* signals (18.2–34.9 ppm *syn*, 14.9–25.7 ppm *anti*; Table 6). Others have reported similar results for the oximes of hydroxylamine (15, 16). PFBOH and PFBOA spectra confirmed the resonances for the aromatic carbons and the resonances for the methylene carbon alpha to the aromatic ring (Table 5). It should be noted that for the aromatic resonances, PFBOH is a better surrogate than PFBOA itself (Table 5).

The IR data are summarized in Table 7. The predominant signals are assigned to carbon–carbon stretching in the aromatic ring, with the most intense absorption appearing around 1505 cm^{-1}. Although carbon–oxygen stretching (1125–1136 cm^{-1}) and nitrogen–oxygen vibrations (927–963 cm^{-1}) were observed, no distinct signal for the carbon–nitrogen double bond could be assigned [generally of weak to medium intensity in the 1471–1689 cm^{-1} region (17)]. Difficulty in assigning the C=N absorption has been reported by others (18). For the PFBOA derivatives, overlap with the carbon–carbon stretching patterns for the aromatic ring is possible (1500–1510, 1521–1532, and 1652–1660 cm^{-1}). Calculation of the percent transmittance for the C=C ring stretch to percent transmittance of the C–H stretch ratio (1650 cm^{-1}/2950 cm^{-1}) showed that the ratio increased as the chain length increased [C1 = 0.37, C2 = 0.79, C7 = 1.84, C10 = 2.34; linear regression equation: $y = 4.62(x) - 1.17$, $r = 0.99$, where y is the ratio of the transmittances of the C=C ring stretch and the C–H stretch at 1650/2950 cm^{-1}, and x is the number of carbon atoms in the original aldehyde]. This equation allows prediction of the IR percent transmittance of other normal-chain derivatives between C1 and C10. Therefore, Fourier transform IR analysis is possible at these 2 wavelengths and at 1505 cm^{-1}.

UV spectrophotometry data at 200, 222, and 264 nm are summarized in Table 8. Data at 222 nm are included because acetonitrile absorbs appreciably below this wavelength. The other 2 wavelengths are the maxima >10^{-5}M for the normal-chain derivatives. Molar absorptivities varied from 491 to 551 L cm^{-1} mol^{-1} at 264 nm for the normal-chain derivatives. The molar absorptivity of PFBOA at 264 nm was 784 L cm^{-1} mol^{-1}, a value higher than for these PFBOA-oximes. The oxime bond probably lowers ring π-electron density. Molar absorptivities at 222 nm are enhanced relative to PFBOA, but are variable at 200 nm. Below 10^{-5}–10^{-6}M, the short wavelength maximum decreased from 200 to 196 nm, and the long wavelength maximum decreased from 264 to 256 for C1 and C2 derivatives; no such effect occurred for C7 and C10 derivatives.

The molar absorptivities of the Gly derivative at 264 and 222 nm were 13 200 and 10 900 L cm^{-1} mol^{-1}, respectively. The maximum wavelengths were 196–200 nm and 250 nm. The molar absorptivity at the latter wavelength was (17 366 ± 690) L cm^{-1} mol^{-1} between 1.25×10^{-5}M and 10^{-4}M. A 254 nm UV detector used commonly in LC would be excellent for the Gly derivative. The absorptivity for PFBOA–Gly appears to be more than additive relative to 2 isolated aromatic rings at 264 nm and may result from super-conjugation of the aromatic systems and the lone pairs of the electrons on the nitrogens in the 2 unsaturated –C=N bonds and on the oxygen atoms (19). The practical lower quantitatable limit is about 10^{-5}M for the normal-chain compounds and 10^{-6}M for the Gly derivative. UV spectrophotometry at 202, 222, and 264 nm is, therefore, feasible for relatively high concentrations. The spectra showed no signs of *syn/anti* isomerism.

Monosubstituted Gly and Propanal Oximes

Three peaks were detected by capillary GC/MS for PFBOA as the limiting reagent relative to Gly alone. Two had molecular ions at 448 (retention times, 33.9 and 34.1 min) and are the unresolved *syn/syn*, *syn/anti*, and *anti/anti* di-PFBOA–Gly

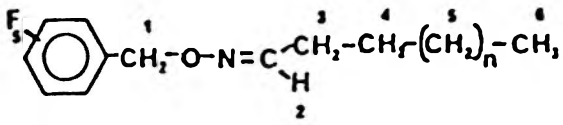
Table 3. Corrected and GC purities calculated from GC/MS data using full scan (50–450 amu) and selected ion monitoring (181, 196, 198, 334 amu) for the PFBOA-oximes of formaldehyde (C1), acetaldehyde (C2), *n*-heptanal (C7), *n*-decanal (C10), and glyoxal (Gly)

	% Purity ± SD	
Starting aldehyde	Corrected purity[a]	GC purity[b]
C1	99.5 ± 2.49	99.9 ± 0.1
C2	99.4 ± 2.49	99.9 ± 0.1
C7	99.3 ± 2.48	99.8 ± 0.1
C10	98.4 ± 2.46	99.4 ± 0.2
Gly	99.2 ± 2.48	99.7 ± 0.2

[a] Accounting for pentafluorobenzyl alcohol and pentafluorobenzyl aldehyde using selected ion monitoring.

[b] Assuming equal MS response factors for the PFBOA-oxime, pentafluorobenzyl alcohol, and pentafluorobenzyl aldehyde using full scan monitoring.

Table 4. ^{1}H NMR spectra of PFBOA-oximes in $CDCl_3$ of formaldehyde (C1), acetaldehyde (C2), *n*-heptanal (C7), and *n*-decanal (C10)[a]



Proton No.	Starting aldehyde	*Syn* Chemical shift, ppm	*Syn* J, Hz	*Anti* Chemical shift, ppm	*Anti* J, Hz	S/A
1	C1[b]	5.19	—	—	—	—
	C2	5.09	—	5.17	—	1.5
	C7	5.10	—	5.15	—	1.8
	C10	5.10	—	5.15	—	1.8
2	C1[b]	7.07 (d)	7.43	6.47 (d)	7.49	—
	C2	7.40 (q)	5.99	6.78 (q)	5.92	1.8
	C7	7.36 (t)	6.11	6.66 (t)	5.93	1.7
	C10	7.35 (t)	6.13	6.67 (t)	5.90	1.7
3	C1	—	—	—	—	—
	C2[c,d]	1.82 (m)	—	—	—	—
	C7	2.14 (m)	6.57	2.27 (m)	5.19	1.7
	C10	2.15 (m)	6.58	2.28 (m)	5.15	1.7
4	C1	—	—	—	—	—
	C2	—	—	—	—	—
	C7[c]	1.43 (m)	6.74	1.43 (m)	6.74	—
	C10[c]	1.44 (m)	6.74	1.44 (m)	6.74	—
5	C1	—	—	—	—	—
	C2	—	—	—	—	—
	C7[c]	1.26 (s)	—	1.26 (s)	—	—
	C10[c]	1.25 (s)	—	1.25 (s)	—	—
6	C1	—	—	—	—	—
	C2	—	—	—	—	—
	C7	0.87 (t)	—	0.87 (t)	—	—
	C10	0.88 (t)	—	0.88 (t)	—	—

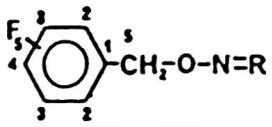
[a] Chemical shift expressed as ppm downfield from tetramethylsilane. Coupling constants are in Hz. *Syn/Anti* ratio (S/A) measured as a function of peak heights: m = multiplet, q = quartet, t = triplet, d = doublet, s = singlet.
[b] Geminal protons, no *syn/anti* isomers.
[c] C3 and C6 are equivalent for this case; C3 is preferred because it is always adjacent to the oxime group apart for C1.
[d] No distinction between *syn/anti* isomers.

oxime derivatives. It is unclear where the *syn/anti* dervative interferes. The third peak (retention time, 16.6 min) is the previously unreported mono-PFBOA–Gly oxime derivative. Although the latter should exist as *syn/anti* isomers, only 1 peak was detected. The spectrum for this peak shows the expected molecular ion of 253, as well as a base peak of *m/z* 181 (Figure 2). Further evidence for a mono-/dioxime mixture came from ^{13}C NMR data, which showed the resonance of a carbonyl functional group at 192.23 ppm downfield from TMS. When Gly reacted in the presence of excess PFBOA, both the 16.6 min retention time peak and the aldehyde resonance in the ^{13}C NMR spectrum disappeared.

When PFBOA was the limiting reagent relative to Gly and propanal, the mono-PFBOA–Gly oxime derivative was again detected. PFBOA–propanal oxime, which also has a molecular ion of 253, eluted at 16.2 and 16.3 min (*syn* and *anti*); the mono-PFBOA–Gly oxime eluted at 16.6 min, and was barely resolvable. When PFBOA was present in excess, the mono-PFBOA–Gly oxime peak disappeared.

Table 9 shows the intensities of the ions ≥181 in the MS of the PFBOA oxime derivatives of acetone, propanal (*syn* and *anti*), and the mono-PFBOA–Gly oxime, all of which have the same molecular weights by low resolution MS. The mass spectra of the PFBOA–acetone oxime (retention time: 15.6 min),

Table 5. ^{13}C NMR spectra in $CDCl_3$ of PFBOA-oximes of formaldehyde (C1), acetaldehyde (C2), *n*-heptanal (C7), *n*-decanal (C10), glyoxal (Gly), and surrogate compounds PFBOA and PFBOH[a]



R = CH_2, $CHCH_3$, $CH(CH_2)_5CH_3$, $CH(CH_2)_8CH_3$

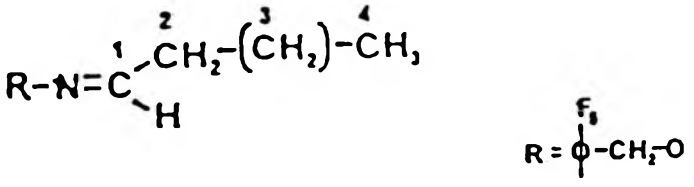
	Chemical shift (ppm) for carbon atom number								
								5	
Starting aldehyde	1	2		3		4		*Syn*	*Anti*
C1	113.51	138.71	141.31	—[b]	145.32	147.06	149.93	65.15	—
C2	114.30	139.90	141.85	143.13	145.91	147.51	150.29	65.41	65.11
C7	114.49	139.73	141.81	143.19	145.26	147.34	150.79	65.28	64.99
C10	114.23	139.04	141.92	142.95	145.76	147.48	150.16	65.29	64.99
Gly	113.62	139.42	142.21	144.04	146.09	146.98	149.72	67.88	66.69
PFBOA[c]	113.91	143.27	145.98	148.18	151.69	151.69	154.49	70.88	—
PFBOH	113.50	139.09	141.88	142.91	145.78	146.98	149.72	52.5	—

[a] Chemical shift expressed as ppm downfield from tetramethylsilane or 2,2-dimethyl-2-silapentane-5-sulfonate (in D_2O).
[b] Signal covered by N=C resonance.
[c] Spectra acquired in D_2O.

PFBOA–propanal oxime, and mono-PFBOA–Gly oxime derivatives differ by the absence of ions at 206 and 236 for the mono-PFBOA–Gly oxime derivative. The relative intensity (R.I.) of ion 206 for PFBOA–acetone oxime is 4.9%, compared with an R.I. of 0.3% for the PFBOA–propanal oxime. Ion 206 may also be used to distinguish these 2 compounds along with the intensities of the 236 ion (R.I. = 3.3 for PFBOA–acetone oxime and >8.0 for the PFBOA–propanal oxime).

Identification of the mono-PFBOA–Gly oxime derivative is important for those attempting to determine if Gly is present in ozonated waters. Because its mass spectrum and its retention time by GC are both similar to the acetone and propanal oximes, the mono-PFBOA–Gly may be misidentified as one of these oximes or as an unknown contaminant. This may cause underestimation of the amount of Gly actually present in an ozonated sample, especially if packed GC columns are used. In

Table 6. ^{13}C NMR spectra in $CDCl_3$ of PFBOA-oximes in $CDCl_3$ of formaldehyde (C1), acetaldehyde (C2), *n*-heptanal (C7), and *n*-decanal (C10)[a]



	Chemical shift (ppm) for carbon atom number						
	1		2		3		
Starting aldehyde	*Syn*	*Anti*	*Syn*	*Anti*	Range		4
C1	141.31[b]	—	—	—	—	—	—
C2	151.41	151.33	18.23[c]	14.90	—	—	—
C7	156.91	155.50	34.45	25.48	32.03 (bs)	29.00 (m)	16.96
C10	156.73	155.63	34.86	25.66	32.27 (bs)	29.05 (m)	17.07

[a] Chemical shift is in ppm downfield from tetramethylsilane: m = center of multiple closely spaced signals, bs = broad singlet.
[b] Geminal protons, no *syn/anti* isomers.
[c] Equivalent to carbon atom 4 for this derivative; C2 is preferred because it is always adjacent to the double bond except for C1 and shows *syn* and *anti* influence.

Table 7. IR spectra in carbon tetrachloride of PFBOA-oxime derivatives of formaldehyde (C1), acetaldehyde (C2), *n*-heptanal (C7), *n*-decanal (C10), and PFBOA and PFBOH

Starting aldehyde	Infrared absorption, cm^{-1}									
	C–H stretch			C–C aromatic stretch	C–C aromatic stretch	C–C aromatic stretch		C–O stretch	N–O stretch	
C1	2895	2981		1657	1527	1509	(s)	1125	927	955
C2	2928	2960		1660	1528	1509	(s)	1134	939	963
C7	2932	2955		1656	1532	1504	(s)	1130	925	946
C10	2925	2976		1657	1521	1502	(s)	1125	939	946
PFBOA[a]	—	—		1652	1530	1510	(s)	1136	920	945
PFBOH[a]	2940	2920	(w)	1660	—	1500	(s)	1126	—	—

[a] From The Aldrich Library of Infrared Spectra: s = strong signal, w = weak signal.

the PFBOA GC/MS method, it is usual to use GC/MS-SIM of ion *m/z* 181 to detect the PFBOA-oxime derivatives. This ion alone cannot distinguish between the PFBOA–acetone oxime, PFBOA–propanal oxime, and mono-PFBOA–Gly oxime derivatives, and retention times do not distinguish the propanal isomers and the mono-Gly derivative clearly. Monitoring of the molecular ion at *m/z* 253 is also not selective. For this reason, SIM of ions 181, 206, and 236 is recommended to distinguish the mono-PFBOA–Gly oxime derivative from the others. The difficulties experienced for Gly are expected for other dicarbonyl compounds such as methyl Gly, a keto-aldehyde.

The presence of the mono-derivative raises the question of the amount of PFBOA necessary to quantitatively derivatize the aldehydes in a sample. Competing matrix constituents may preferentially react with PFBOA, leaving an insufficient amount to react quantitatively with the known aldehydes present. This matter is considered in the next section.

Gly, a direct-acting mutagen (20) known to be present in ozonated waters (3–6), is an ozonolysis by-product of naphthoresorcinol (21). Chlorination of Gly results in mutagenic activity in the *Salmonella* reverse mutation assay (22). Ingested Gly is also genotoxic in rats (23).

Method Development for Aldehyde Determination in Water

Using the published method as a basis, experiments were divided into those optimizing recoveries of known concentrations of PFBOA oximes in hexane after extraction and washing and those concerned with the reaction efficiency between aldehydes and PFBOA. For the first objective, whether the first and final sulfuric acid washings were necessary was investigated. Addition of 50 µL 18N sulfuric acid to 5 mL aqueous solution containing 1–10 mg PFBOA/mL reduced the PFBOA amount found by GC to 3% compared with no washing; washing with 5 mL 0.1N sulfuric acid decreased the original PFBOA extracted to 0.7%, as did washing with a combination of 50 µL sulfuric acid + 5 mL 0.1N sulfuric acid. However, the recovery of PFBOA aldehyde oximes was also affected. PFBOA oximes at molar concentrations in hexane equivalent to starting aqueous aldehyde concentrations were washed separately with 5 mL water solutions containing 50 µL concentrated sulfuric acid, with 5 mL 0.1N sulfuric acid, and with the combination treatment. The last 2 treatments caused significant losses of the C1 oxime derivative in hexane relative to unwashed samples (18 and 31%, respectively). Only the combination treatment caused significant losses of C2 (13%) and C10 (23%) oxime derivatives. Thus, the second sulfuric acid washing was deleted because of PFBOA oxime losses and because the GC resolution between PFBOA free base and the C2 PFBOA oxime was adequate up to 10 mg PFBOA/mL. At 20 mg/mL, the PFBOA interfered with the C2 oxime quantitation. The 10 mg/mL concentration of PFBOA was selected for further experimentation because it was the highest concentration that could be used without causing substantial interference with C2 PFBOA

Table 8. Molar absorptivities calculated for selected UV-vis spectra wavelengths of selected PFBOA-oximes (1.25×10^{-5}M–1.0×10^{-4}M) in acetonitrile of formaldehyde (C1), acetaldehyde (C2), *n*-heptanal (C7), *n*-decanal (C10), glyoxal (Gly), and surrogate compound PFBOA

Starting aldehyde	Molar absorptivity ± SE, L/(mole cm)		
	200 nm	222 nm	264 nm
C1	13800 ± 550	2970 ± 140	551 ± 20
C2	13900 ± 500	3090 ± 60	517 ± 2.9
C7	10200 ± 360	3680 ± 230	491 ± 20
C10	10700 ± 370	3900 ± 160	533 ± 4.8
Gly	13600 ± 540	10900 ± 780	13200 ± 820
PFBOA[a]	11400 ± 430	2457 ± 50	784 ± 20

[a] Measured in ASTM Type I water.

Table 9. Relative intensities of mass spectral ions ≥ m/z 181 of the oximes of PFBOA–acetone, PFBOA–propanal, and mono-PFBOA–glyoxal

	Relative intensity, % ± SD for the PFBOA oximes[a]		
m/z	Acetone	Propanal[b]	mono-PFBOA–glyoxal
181	100	100	100
	—	100	—
182	7.0 ± 0.1	7.1 ± 0.2	7.0 ± 0.2
	—	6.9 ± 0.2	—
195	4.3 ± 0.1	3.7 ± 0.1	3.7 ± 0.2
	—	3.8 ± 0.2	—
206	4.9 ± 0.5	0.3 ± 0.0	ND[c]
	—	0.2 ± 0.0	—
223	4.6 ± 0.6	5.5 ± 0.4	0.2 ± 0.0
	—	3.3 ± 0.2	—
236	3.3 ± 0.3	8.6 ± 0.1	ND[c]
	—	15.4 ± 0.5	—
253	5.3 ± 1.1	1.2 ± 0.1	0.3 ± 0.1
	—	1.2 ± 0.1	—

[a] $n = 3$ injections of each.
[b] *Syn* and *anti* isomers.
[c] ND = not detected.

oxime quantitation and without having to wash a second time with sulfuric acid to eliminate that interference.

It was then shown that 3 extractions are required for quantitative recovery for all PFBOA derivatives, because the recovery for 1 extraction varied between 82 and 90%, and the recovery for 2 extractions ranged between 97 and 99%. The recovery for 3–5 extractions was always 100%. Varying the concentration of PFBOA between 1 and 20 mg/L did not affect hexane extraction recovery of PFBOA oxime derivatives in distilled water. The addition of 100 µL sodium thiosulfate solution produced the same recoveries of oxime esters as when no sodium thiosulfate was present. Thus, once formed and extracted into hexane, the PFBOA oximes are stable. Because the Gly–PFBOA derivative tends to precipitate at room temperature and in the refrigerator in hexane, hexane solutions that are not freshly extracted must be shaken well, warmed to 40°C, and ultrasonicated 2 min before injection. This procedure was adopted before all injections. The use of the internal standard method for PFBOA derivative extractions is valid for 2 extractions and above because 90–95% of the DFB internal standard was extracted with 1 hexane extraction and 100% with 2–5 extractions.

For optimization of reaction conditions, pH, reaction temperature, reaction time, and PFBOA concentration were investigated. First, an aqueous solution of 4.0×10^{-3}M PFBOA was titrated with 1.5×10^{-3}M sodium hydroxide. The pK_a was between 3.0 and 3.2, suggesting that protonation dominated below this pH. The equivalence point was between 6.8 and 7.0, where the hydrochloride part of PFBOA was fully neutralized. The initial pH was 2.89. The rate and equilibria of the reaction between hydroxylamine hydrochloride and aldehydes may be pH-dependent because it is known that the *syn* isomer is the thermodynamically stable isomer of oxime O-methyl ethers; the kinetic product, the *anti* isomer, is formed first (13, 14). This time-dependence of kinetic/thermodynamic product probably accounts for changes in the NMR resonance *anti/syn* intensity ratios of freshly synthesized PFBOA derivatives.

For distilled water experiments, the pH after the addition of 1 mg PFBOA/mL was 2.5–3.8. After addition of 50 µL sulfuric acid, the pH was 1.1–1.5. Spiked aldehydes at various pH values and buffers were evaluated in the presence of 100 µL sodium thiosulfate with 50 µL sulfuric acid added after 2 h reaction at 25°C with 10 mg PFBOA/mL before extraction with three 1 mL portions of hexane (Table 10). The buffers were added before PFBOA reaction and included pH 4 phthalate (400 µL), pH 7 phosphate (500 µL), and pH 10 carbonate (500 µL). C1 and C2 PFBOA oxime derivatization did not vary significantly at all pH values in comparison to derivatization when no added buffer was present, although yield precision suffered at pH >3, with coefficients of variation increasing up to 15% (Table 10). The yields for C7, C10, and Gly PFBOA oximes were decreased significantly at pH 3 and above to 51–68% of those at pH 1–3. The next step with the above procedure was to adjust the initial pH with HCl because PFBOA already was present as the hydrochloride salt, and this eliminated any initial effects of sulfate ion. Significant decreases of 15–25% for all aldehyde PFBOA oximes occurred compared

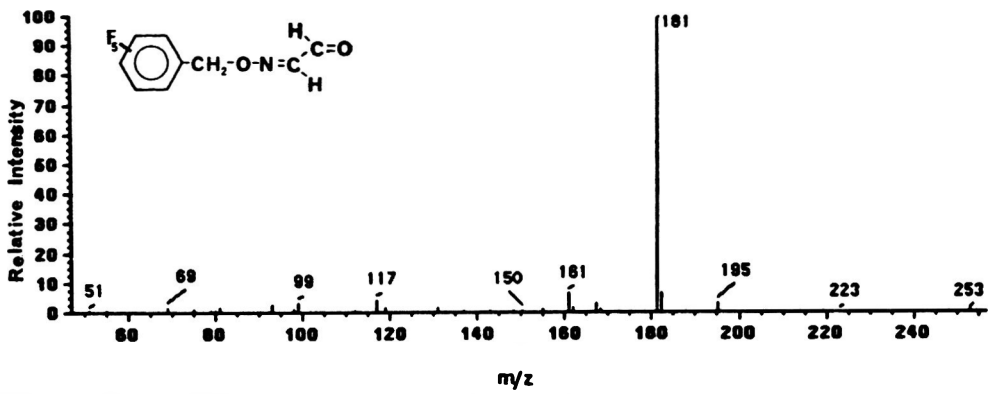


Figure 2. Electron impact (70 eV) mass spectrum of the mono-PFBOA–glyoxal oxime, molecular weight = 253.

Table 10. pH values measured during the various stages of the 10 mg PFBOA/mL reaction with aldehydes in 5 mL distilled water (in mg/mL: C1, 333; C2, 202; C7, 518; C10, 691; and glyoxal, 68), and in the presence of different buffers

	pH after addition				
			Buffer pH, 500 µL		
After addition, chemical added	18N H_2SO_4, 50 µL	PFBOA	4	7	10
Distilled water + aldehyde	5.9	5.9	5.9	5.9	5.9
0.1N $Na_2S_2O_3$, 100 µL	5.6	5.6	5.6	5.6	5.6
18N H_2SO_4, 50 µL[a]	1.3	1.3	1.3	1.3	1.3
Buffer	—[b]	—	4.1	7.4	10.3
PFBOA	1.4	2.8	3.3	5.1	9.4
After 2 h reaction, at 25°C	1.1	2.3	2.9	4.9	9.3
18N H_2SO_4, 50 µL	1.1	1.2	1.3	1.3	1.3
Hexane extraction, three 1 mL portions	1.0	1.1	1.1	1.1	1.1

[a] pH of 1.3.
[b] —, No addition.

with yields observed for initial pH values of 0.9–2.0 when the reaction pH was 0.5 or 3.0. The yields of the long-chain PFBOA oxime derivatives were affected the most, in agreement with the results for the pH experiments with buffers. Thus, 25 µL HCl was added to the distilled water solution of aldehydes after the addition of thiosulfate and before PFBOA was added. In the field, after waters of different buffering capacity were sampled, the pH would have to be adjusted immediately into the 0.9–2.0 range with the appropriate volume of HCl.

The next step was to optimize the temperature for the reaction itself. The modified reaction sequence above (with the extra HCl) was repeated for 2 h at 25°C, 2 h at 70°C, 30 s in a microwave oven at 80–82°C, and overnight (16 h) at 25°C. The yields of the C1 and C2 PFBOA derivatives were not affected by any treatment. In the non-optimal techniques, the derivatization continued slowly after extraction into hexane for the long-chain aldehydes in the presence of excess PFBOA extracted into the hexane. The microwave treatment was optimal for C7, C10, and Gly PFBOA oxime derivatization, generally giving yields up to 50% higher than for the other techniques. Thus, the microwave technique was chosen as the optimal heating method, with the advantages of extremely short reaction times and no variation of yields on storage. However, if aldehydes were produced by thermal decomposition or ozonated water under these conditions, this would be an artifact. Such an artifact has been described for formaldehyde by Yamada and Somiya in freshly ozonated waters (24). Because excess ozone conditions are unlikely to be present on sampling from a redistribution system or after a chlorination step, this artifact is probably important for samples taken directly after the ozonolysis step.

Direct Comparison of Optimized and Literature Methods for Various Waters

Table 11 presents the results of the yields of reaction products for the 1-extraction/2-sulfuric acid addition technique relative to the 3-extraction/HCl addition/microwave method for various types of waters. The optimized method has better precision and more yields >80% than the published method, especially for the long-chain PFBOA derivatives.

The optimized method was superior 5/5 times for C10 aldehyde determinations, 3/5 times for Gly, 2/5 times for C2, and 1/5 times for C1, and was no different for the C7 derivative. Of a possible 25 times, the optimized method showed >80% recoveries for all. In contrast, the published method (4) provided 14/25 (56%) acceptable recoveries >80%. Because the recoveries for the C1, C2, C7, and C10 normal aldehydes detected in ozonated waters are >80% by the optimized method, the recoveries of all the normal aldehydes of shorter chain length than C11 are also expected to be >80%. Because the recovery of the Gly derivative, for which steric hindrance might be a factor, was quantitative, it is expected that the recoveries for other dialdehydes should also be quantitative as long as the optimum pH, temperature, and excess PFBOA are used for the reaction. Thus, the optimized method is a general method for quantitation of aliphatic mono- and dialdehydes in aqueous solution. The occurrence of the mono-PFBOA–Gly derivative illustrates an artifact which, if not recognized, would lead to underestimation of Gly. This also applies to other dialdehydes and difunctional carbonyl compounds such as the keto-aldehyde methyl Gly. The occurrence of artifacts by aldehyde formation from thermal decomposition of water components at 80°C, or by the presence of excess ozone in the water, is a potential positive interference.

The poor performance of the published method (4) for the SRM 1643b and Los Angeles tap water is noteworthy. GC/MS showed that no PFBOA peak was detected in analysis of SRM 1643b samples, indicating that the major problem relative to matrix effects was to ensure excess PFBOA for reaction. The PFBOA peak was decreased relative to distilled water for the Los Angeles tap water sample. In field samples, many aldehydes, ketones, and other species may be consuming PFBOA, so that appearance of the PFBOA peak on the chromatogram is assurance that quantitative reaction probably occurred. The second sulfuric acid washing in the published method would

Table 11. Comparison of the efficiencies of 1-extraction method with 3-extraction method for the same water types spiked with 68–700 ppb aldehydes in triplicate (arithmetic mean ± 1 SD)[a]

Water type	Method	Method efficiency, % for PFBOA oxime of aldehyde				
		C1	C2	C7	C10	Glyoxal
Distilled	3	94 ± 6	99 ± 7	88 ± 9	100 ± 7	95 ± 10
	1	88 ± 3	88 ± 4	83 ± 6	47 ± 1[b]	76 ± 4[b]
SRM 1643b	3	109 ± 3	94 ± 8	95 ± 7	81 ± 7	92 ± 10
	1	44 ± 5[b]	73 ± 8[b]	100 ± 4	67 ± 4[b]	45 ± 4[b]
SRM 2694a-I	3	96 ± 5	100 ± 9	96 ± 8	94 ± 3	83 ± 10
	1	89 ± 3	89 ± 4	96 ± 3	66 ± 4[b]	87 ± 10
SRM 2694a-II	3	98 ± 8	99 ± 10	98 ± 5	91 ± 7	83 ± 6
	1	86 ± 7	92 ± 1	100 ± 3	63 ± 5[b]	91 ± 5
LA tapwater	3	101 ± 8	81 ± 12	98 ± 10	100 ± 6	92 ± 10
	1	90 ± 13	69 ± 4[b]	96 ± 8	41 ± 4[b]	30 ± 10[b]

[a] The 3-extraction 10 mg PFBOA/mL solution technique with 1 addition of sulfuric acid is denoted by "3," and the 1-extraction 1 mg PFBOA/mL solution method with 2 additions of sulfuric acid is denoted by "1." Concentrations were in ng/mL: C1, 333; C2, 202; C7, 518; C10, 691; and glyoxal, 68.

[b] $p < 0.025$ with respect to the optimized method.

not allow this interpretation; absence of the PFBOA peak does not necessarily imply inadequate excess of PFBOA during reaction. This is especially important for sampling ozone contactor effluents, because the PFBOA demand is expected to be greater than for waters from the redistribution system.

The 2 methods gave equivalent results for both rainwater SRM samples except with C10, for which the optimized method was definitely superior. The methods were equivalent for both SRM rainwaters and distilled water for C1, C2, and C7 aldehydes, but not for C10 or Gly.

The optimized method has been shown to be valid at the following highest concentrations of cations and anions simultaneously present in ng/g from the SRM and distilled water concentrations: Ag, 10; Al, 23; As, 49; B, 94; Ba, 44; Be, 19; Bi, 11; Ca, 34 000; Cd, 20; Co, 26; Cr, 19; Cu, 22; Fe, 99; K, 3000; Li, 5; Mg, 15 000; Mn, 28; Mo, 85; Na, 7900; Ni, 49; Pb, 24; Se, 10; Si, 3100; Sr, 227; Th, 8; V, 45; Zn, 66; ammonium ion, 1100; chloride, 4000 (accounting for added HCl and PFBOA, also); fluoride, 110; nitrate, 31 000; and sulfate, 11 000. Most drinking waters contain concentrations that are below these values. The pH must be adjusted between 0.9 and 2.0 and the appropriate heating conditions used for efficient reaction. All the normal-chain aldehydes can be resolved from one another on the GC column by using chromatographic conditions cited in the present study. Work is underway to assess ketone derivatization with PFBOA, including the keto-aldehyde, methyl Gly.

This method is one of the few available for aliphatic mono- and dialdehydes that is sensitive to the pg level and yet quantitative. Rival methods such as (2,4-dinitrophenyl)hydrazine derivatization (25) are not sensitive enough, and 2-(hydroxymethyl)piperidine derivatization (26) is unvalidated. Screening by GC/ECD is also possible.

To ensure a good probability of assurance for environmental measurements, we recommend the use of SRM samples for method ruggedization before field measurements begin.

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PESTICIDES AND INDUSTRIAL CHEMICAL RESIDUES

Diphasic Dialysis: A New Membrane Method for a Selective and Efficient Extraction of Low Molecular Weight Organic Compounds from Aqueous Solutions

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A new membrane technique is described for the extraction of low molecular weight organic compounds from a wide range of substrates. With this system, the organic compounds of interest contained in the sample pass through the membrane toward the organic solvent, while other substances of higher molecular weight are excluded by the membrane. This procedure allows the extraction or the elimination of toxic substances with a yield of 90%.

Public health officials show increasing concern at the presence of pollutants in the environment and in the food chain (1, 2). Most of these pollutants (e.g., pesticides, mycotoxins, pharmaceuticals, and residues from chemical industries) are organic compounds of relatively low molecular weight (MW) that are soluble in organic solvents. Control techniques usually require an initial extraction step followed by the purification and concentration of the extracts before their identification and quantification by different physical, chemical, immunological, or biological procedures (3, 4).

The extraction step is normally performed with different organic solvents, alone or in combination with a small amount of an aqueous solution containing salts or acids. A sample preparation procedure is also used to eliminate interfering compounds. Finally, the extraction is performed with a single organic solvent in which the compound under study is very soluble (3, 4). These procedures are usually expensive and very time-consuming (5).

Simpler and more accurate methods, which permit automation, are now being developed (6–8). The technology of membranes (dialysis, ultrafiltration, reverse osmosis, etc.) is also being used more and more for recovering and analyzing these substances (9–11). The use of dialysis membranes allows, for example, the separation of compounds of high MW from those

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of low MW by molecular exclusion in a dialysis system (8). Theoretically, a static dialysis for an infinite time interval (with equal sample and recipient solvent volumes) will cause 50% of the substance of interest in the sample to permeate into the solvent recipient (9, 10); this efficiency can be improved by increasing the ratio of the volumes or by varying other factors such as the temperature, the exchange surface, or the shaking speed in some cases, or by applying electrodialysis.

In our laboratory, we took advantage of 2 common properties of organic contaminants, i.e., their solubility in organic solvents and their low molecular weight, to develop a semipermeable membrane technology for the extraction of such substances from different substrates. With this system, the organic compounds of interest pass through the membrane toward the organic solvent, while other substances of higher MW are retained by the membrane. The driving force for movement of substrate from the aqueous phase through the membrane into the organic solvent is the partition coefficient, which is a function of both the leaving tendency (fugacity) from the aqueous phase and the solubility in the organic phase of the substrate. The movement through the membrane is a process in which the 2 phases are interdependent.

This method can be applied to detection, extraction, or elimination of low MW organic compounds from different samples. We used the method to extract or eliminate 90% of certain toxic substances without increasing the volume of the organic solvent. This is because distribution of the analyte on both sides of the membrane depends on the partition coefficient between the aqueous phase and the solvent and not on the osmotic pressure, as in the traditional dialysis.

Experimental

Principle

The solvent used to extract the organic substance under study from aqueous substrates is introduced in a dialysis tubing of regenerated cellulose with a molecular exclusion size of 10 000 daltons, previously hydrated by submerging it in water. The dialysis bag is placed into a flask containing the substratum, and exchange is promoted by shaking. The contents of the dialysis tubing are then dehydrated with anhydrous sodium sulfate and concentrated by using a rotary evaporator. The resulting sample is ready for identification and quantitation by thin-layer chromatography (TLC) or liquid chromatography.

Materials

(**a**) *Dialysis tube.*—Visking dialysis tubing 20/32 (Serva, Feinbiochemical, Heidelberg, Germany).

(**b**) *Solvents and reagents.*—Chloroform, methanol, acetone, isopropanol, anhydrous sodium sulfate, ethyl acetate, toluene, 90% formic acid, acetic acid, methylene chloride, HCl. All were analytical grade.

(c) *Aflatoxin M_1 standard.*—10 μg/mL (Sigma Chemical Co., St. Louis, MO 63178). Dissolved in chloroform, deep-frozen (–30°C), and calibrated photometrically (*Official Methods of Analysis*, **979.44**).

(**d**) *Patulin standard.*—50 μg/mL (Sigma Chemical Co.). Dissolved in chloroform, deep-frozen (–30°C), and calibrated photometrically (*Official Methods of Analysis*, **974.18**).

(**e**) *3-Methyl-2-benzothiazolinonehydrazone (MBTH) hydrochloride.*—Kept under refrigeration, used in 0.5% aqueous solution, and prepared on day of use.

(**f**) *Clenbuterol standard.*—50 μg/mL (Sigma Chemical Co.). Dissolved in ethyl acetate and deep-frozen (–30°C).

(**g**) *Bratton-Marshall reagent.*—For TLC determination of clenbuterol. Composed of 2 solutions: Solution I, 1 g $NaNO_2$ dissolved in 20 mL distilled water and diluted to 100 mL with concentrated HCl–ethanol (17 + 83); Solution II, 1 g *N*-(1-naphthyl)ethylenediamine dissolved in 10 mL distilled water, and diluted to 100 mL with ethanol. Prepared on day of use.

(**h**) *Heptachlor standard.*—10 μg/mL (Chemical Service). Dissolved in acetone and deep-frozen (–30°C).

Samples

Milk samples were spiked with aflatoxin M_1 at 0.05 μg/L, apple juice samples with patulin at 100 μg/L, urine samples with clenbuterol at 100 μg/L, and water samples with heptachlor at 2.5 μg/L.

Extraction

A 70 mL aliquot of the extraction solvent (chloroform, ethyl acetate, or hexane) was placed in a previously hydrated dialysis tubing 60 cm long with ca 470 cm^2 of exchange surface. Then, 50 mL of the sample was placed in a 1 L flask, the dialysis tubing containing the extraction solvent was introduced, and the sample was extracted by shaking (120 rpm) 5 h at 37°C. In experiments with milk, these samples were shaken with 50, 250, and 1000 mL solvent for 1, 2, 3, 5, 7, and 14 h.

After the shaking process, the contents of the dialysis tubing were decanted into a funnel, the aqueous layer was removed, and the organic layer was dried with anhydrous sodium sulfate on filter paper. The extract was then placed in a round-bottom flask and concentrated on a rotary evaporator to 200 μL (milk and urine) or to 500 μL (apple juice and water).

Determination of Aflatoxin M_1

Aflatoxin M_1 was detected by TLC using high resolution 10 × 10 cm plates without fluorescence indicator (Merck No. 5721). The plates were developed in an unsaturated chamber with chloroform–methanol (90 + 10). Aflatoxin M_1 was quantitated with a densitometer (Camag TLC/HPTLC Scanner II) equipped with Cats 3 software, using a UV lamp with excitation wavelength of 350 nm.

Determination of Patulin

Patulin was detected by TLC using 20 × 20 cm plates without fluorescence indicator (Merck No. 5721). The plates were developed in an unsaturated chamber with toluene–ethyl acetate–chloroform–90% formic acid (70 + 50 + 50 + 20). The developed plates were air-dried and sprayed with 0.5% MBTH solution until moist and then heated 15 min in an oven at 130°C. Patulin was quantitated with a densitometer (Camag

Table 1. Recovery of aflatoxin M_1, patulin, clenbuterol, and heptachlor from artificially contaminated samples (milk, apple juice, urine, and effluent water)

Product	Compound	Organic solvent	No. sample	Added, μg/L	Rec., μg/L		Rec., %	
					Av.	Range	Av.	CV
Milk	Aflatoxin M_1	Chloroform	8	0.05	0.048	0.038–0.055	96.5	12.7
Apple juice	Patulin	Ethyl acetate	4	100	62.5	60–65	62.5	3.3
Urine	Clenbuterol	Ethyl acetate	3	100	82.5	78–87	82.5	5.4
Water	Heptachlor	Hexane	2	2.50	2.32	2.27–2.37	92.8	2.2

TLC/HPTLC Scanner II) equipped with Cats 3 software, using a UV lamp with excitation wavelength of 360 nm.

Determination of Clenbuterol

Clenbuterol was detected by TLC using high resolution 10 × 10 cm plates without fluorescence indicator (Merck No. 5631). The plates were developed in an unsaturated chamber with ethyl acetate–methanol–acetic acid (80 + 10 + 10). The developed plates were air-dried and sprayed with Solution I of the Bratton-Marshall reagent until the silica gel was transparent. The plates were air-dried again 10 min, sprayed with Solution II until the silica gel was transparent, and heated 5 min in an oven at 110°C. Clenbuterol was quantitated with a densitometer (Camag TLC/HPTLC Scanner II) equipped with Cats 3 software, using a tungsten lamp with excitation wavelength of 520 nm.

Determination of Heptachlor

Heptachlor was detected by TLC using high resolution 10 × 10 cm plates with fluorescence indicator (Merck No. 5628). The plates were developed in a saturated chamber with methylene chloride.

Heptachlor was quantitated with a densitometer (Camag TLC/HPTLC Scanner II) equipped with Cats 3 software, using a deuterium lamp with excitation wavelength of 225 nm.

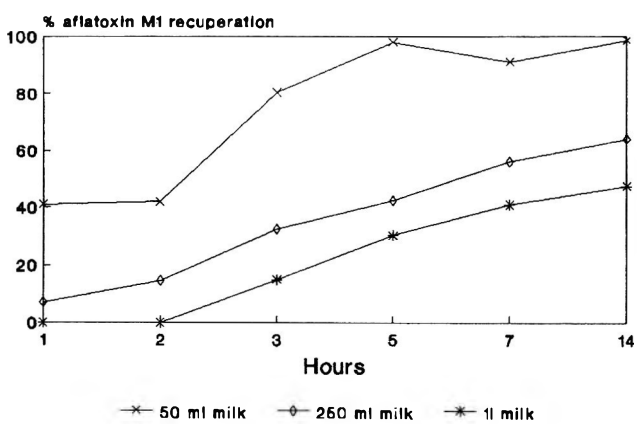

Figure 1. Recovery of aflatoxin M_1 from different volumes of milk spiked at 0.05 μg/L and extracted with 70 mL chloroform at several time intervals in a shaking system.

Results and Discussion

As shown in Table 1, a number of organic substances with different molecular characteristics can be extracted and purified by the system reported here. In this study, 4 representative types of toxic compounds, including 2 toxic fungal metabolites (aflatoxin M_1 and patulin), a pesticide (heptachlor), and a pharmaceutical used fraudulently in the fattening of animals for meat (clenbuterol), were determined. We chose different substrates to test the versatility of the method: a food of animal origin (milk), a food of vegetable origin (apple juice), an organic fluid (urine), and water (in motion).

A critical factor in this technique is the organic solvent used. It should be immiscible with the aqueous solution to be analyzed so that the passage of the solvent toward the sample is minimized; therefore, problems of eliminating the treated sample are minimized. Equally important is the lack of solubility between both liquids, so that leakage of the organic solvent is very difficult; this consideration is of maximum interest, because it minimizes contamination of the tested samples with the organic solvent.

Another critical factor to bear in mind is that the analyte should be more soluble in the organic solvent than in the aqueous sample in which it is originally contained. As explained previously, the yield of the extraction will be a function of the partition coefficient of the sample and the selected solvents. Under the best conditions, the elimination of pollutants or the extraction of compounds of analytical or industrial interest can reach a yield of 90% (Table 1).

The extraction of the analyte is another factor to take into account. When the organic solvent has been properly selected in accordance with the substrate from which the analyte is extracted, the number and quantities of interfering substances can be controlled and minimized. In this sense, one can also modify the physical and chemical conditions of the aqueous sample (e.g., pH), increase or decrease the solubility of the analyte in the aqueous phase, and, thus, modify the partition equilibrium.

Another important parameter in the optimization of the technique is the ratio of the aqueous sample volume to that of the organic solvent. Additional factors are the membrane surface area and the time of extraction.

Figure 1 represents the results obtained by applying our method to the extraction of aflatoxin M_1 from milk spiked at 0.05 ppb at different volumetric ratios and times. Under the worst conditions (sample:solvent volumetric ratio of 1000:70),

a 14 h extraction produced the same yield as a traditional dialysis procedure with a volumetric ratio of 1:1, i.e., 45%. When this ratio was decreased to 250:70 for 7 h, the yield was about 50%; with the best conditions (ratio 50:70), the percentage of extraction rose above 98% in only 5 h. In these experiments, we used an exchange surface membrane with an area of approximately 470 cm^2 and a shaking system.

The yield of the system would be greater if a continuous extraction system and countercurrent flow were used and if conditions of pressure and surface exchange were varied.

Applications

The methodology described here may be applied for other than analytical purposes. Extraction for elimination of environmental pollutants or for products of industrial interest can also be considered. Among the possiblities, we cite the following potential applications: Analytical determinations, as shown in this paper; extraction of metabolites of industrial interest (hormones and prostaglandins) from animal residues; control of the level of contamination in the networks of distribution and canalization of water via industrial waste or others; and extraction and elimination of residues and pollutants from water effluents.

Acknowledgments

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Liquid Chromatographic Determination of Pesticides in Finished Drinking Waters: Collaborative Study

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A joint U.S. Environmental Protection Agency (EPA)/AOAC International collaborative study was conducted on EPA National Pesticide Survey Method 4, *Determination of Pesticides in Finished Drinking Water by High Performance Liquid Chromatography with an Ultraviolet Detector*, to determine mean recovery and precision for analyses of 18 pesticides and metabolites in reagent water and finished drinking waters. The study design was based on Youden's nonreplicate plan for collaborative studies of analytical methods. The waters were spiked with 18 pesticides and metabolites at 6 concentration levels, prepared as 3 Youden pairs. Ten volunteer laboratories extracted the spiked test waters with methylene chloride, performed a solvent exchange with methanol, and analyzed an aliquot of each extract by liquid chromatography using an ultraviolet detector at 254 nm. Results were analyzed using an EPA computer program that calculated recovery and precision for each of the 18 compounds and compared method performance between water types. The method was judged useable for all analytes tested. Recoveries were good for 16 of the analytes. The method exhibited poor recovery but acceptable between-laboratory precision for metribuzin DADK and metribuzin DK. The method performance statistics for carbofuran phenol and linuron were calculated to be significantly different for reagent water and finished drinking water. Similar statistical differences were observed for metribuzin DADK, but these effects were not considered of practical significance due to the poor recovery (<25%) in both waters. In reagent water, the overall relative standard deviation (RSD_R) range was 7.8–24.1% for all 18 compounds and 5.5–38.6% in finished drinking water. The single-analyst relative standard deviations (RSD_r) range was 5.7–17.6% in reagent water and 4.0–18.7% in finished drinking water. The method has been adopted first action by AOAC International.

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The recommendation was approved by the General Referee, Committee Statistician, Committee Safety Advisor, and by the Committee on Environmental Quality. The method has been adopted first action by the Official Methods Board at their January 1992 meeting. Association actions will be published in "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76**, January/February issue.

The widespread contamination of vulnerable ground water supplies by pesticides and herbicides became a major concern to the U.S. Environmental Protection Agency (EPA) in the early 1980's. In the fall of 1983, EPA was charged by the Congress of the United States to monitor drinking water supplies in the United States to assess the degree of contamination by these compounds. As a result, EPA initiated the National Pesticide Survey (NPS) to provide a comprehensive assessment of water contamination in private and public wells in the 50 states. NPS Method 4 (1) was developed specifically to analyze temperature labile pesticide compounds, using liquid chromatography (LC) and ultraviolet (UV) detection at 254 nm.

EPA's Environmental Monitoring Systems Laboratory at Cincinnati, OH (EMSL-Cincinnati), develops or selects analytical methods and provides quality assurance (QA) support for the Agency's water and wastewater regulations. The Quality Assurance Research Division (QARD), EMSL-Cincinnati, provides the QA support to establish the reliability and legal defensibility of water and wastewater data collected by the Agency, the state regulating authorities, the private sector, and commercial laboratories that perform compliance analyses. Among QARD's activities is the evaluation of selected analytical methods through interlaboratory validation studies. This

paper describes the results of a joint EPA/AOAC collaborative study of NPS Method 4.

The study was conducted under the direction of QARD. As primary contractor to QARD, The Bionetics Corp. was responsible for the preparation, analysis, and distribution of sample concentrates, instructions to collaborators, report forms, review of the returned data, and submission of the final report. QARD statistically evaluated the raw data using a computer program, Interlaboratory Method Validation Study (IMVS) (2), designed for these studies.

The objective of this study was to characterize multilaboratory performance of NPS Method 4 in terms of recovery, overall and single-analyst precision, and the effect of water type on recovery and precision for the 18 compounds investigated in the method.

Collaborative Study

The study design was based on Youden's nonreplicate design for collaborative evaluation of overall precision, single-analyst precision, and mean recovery for analytical methods (3). Two samples with similar concentrations of each of the 18 analytes were analyzed as Youden pairs at each of 3 levels to provide data for estimating single-analyst precision. The 18 analytes were divided into 2 mixes (A and B) to minimize resolution problems. The collaborating laboratories were directed to extract the spiked samples, analyze each extract, and report a value for each of the analytes present. The evaluation of spiked reagent water analyses demonstrated the proficiency of the method on a sample free of interferences; that of the spiked drinking waters demonstrated the suitability of the method for regulated drinking water and permitted a comparison of results with reagent water.

Spiking solutions, calibration standards, and quality control samples were prepared and heat-sealed in ampules, each containing approximately 1.5 mL solution. Prior to distribution, the ampuled solutions were analyzed against standards freshly prepared from neat materials. At the completion of the study, the ampuled solutions were analyzed again to verify the stability of the solutions over the time of the study.

Each of the 16 participating laboratories received 24 sample ampules (6 concentrations for each of 2 waters for 2 groups of target compounds), 4 calibration standard concentrates, 3 internal standard concentrates, 3 surrogate standard concentrates, 4 quality control samples with acceptance limits, report forms, and a questionnaire. The analysts were instructed to analyze the samples in strict accordance with the method (1) and to complete the analyses within 60 days from receipt of the samples.

992.14 Pesticides in Water—Liquid Chromatography Method

First Action 1992

(Applicable to determination of 18 pesticides and metabolites in finished drinking water in low-to-mid ppb range)

Method Performance:

Collaborative study showed method acceptable for these analytes: atrazine dealkylated, barban, carbofuran phenol, cyanazine, diuron, fenamiphos sulfone, fenamiphos sulfoxide, fluometuron, 3-ketocarbofuran phenol, linuron, metribuzin DA, metribuzin DADK, metribuzin DK, neburon, pronamide metabolite, propanil, propham, and swep. In reagent water, RSD_R range was 7.8%–24.1% for all 18 compounds; in finished drinking water, RSD_R range was 5.5%–38.7%. In reagent water, metribuzin DADK and metribuzin DK exhibited very poor extraction efficiency (<50%) but acceptable precision, RSD_R 17.2 and 17.5%, respectively. In finished drinking water, their RSD_R values were 29.3 and 28.8%, respectively. *See* Table **992.14A** of method performance data.

(*Caution*: See safety notes on organic solvents and special chemical hazards.)

A. Principle

Measured volume of sample is extracted with CH_2Cl_2 by shaking in separatory funnel or mechanical tumbling in bottle. CH_2Cl_2 extract is separated, dried with anhydrous Na_2SO_4, solvent-exchanged to methanol, and concentrated. Pesticides are separated and measured by liquid chromatography using ultraviolet (UV) detector.

B. Apparatus

(**a**) *Grab sample bottle.*—1 L borosilicate glass, with TFE-fluorocarbon lined screw caps (Wheaton Media/Lab bottle 219820 is suitable). Extract contaminants from cap liners overnight with methanol before use.

(**b**) *Separatory funnel.*—2 L borosilicate glass, with TFE-fluorocarbon stopcock and ground-glass or TFE-fluorocarbon stopper.

(**c**) *Tumbler bottle.*—1.7 L low extractable borosilicate glass, with TFE-fluorocarbon lined screw cap (Wheaton Roller Culture Vessel is suitable). Cap liners are cut from TFE-fluorocarbon sheets (Pierce Catalog No. 012736 is suitable); remove contaminants from liners by soaking overnight in methanol before use.

(**d**) *Erlenmeyer flasks.*—500 mL borosilicate glass.

(**e**) *Drying column.*—400 mm × 19 mm id, with coarse fritted disk.

(**f**) *Concentrator tube, Kuderna-Danish (K-D).*—10 or 25 mL borosilicate glass, graduated, with 19/22 top. Check calibration of concentrator tubes at volumes used in method. Use ground-glass stoppers to prevent evaporation.

(**g**) *Evaporative flask, K-D.*—500 mL borosilicate glass, 20/40 top, 19/22 bottom, capable of attachment to concentrator tube with spring connectors.

(**h**) *Snyder column, K-D.*—3-ball macro, 218 mm, 24/40 bottom.

(**i**) *Snyder column, K-D.*—2-ball micro, 170 mm, 19/22 bottom.

(**j**) *Vials.*—Glass, 5–10 mL capacity with TFE-fluorocarbon lined screw caps.

(**k**) *Syringes.*—Disposable glass, frosted tip, 2.5 mL (B-D Glaspak No. 5291 is suitable).

Table 992.14A. Performance parameters for 18 pesticides in reagent water and finished drinking waters[a]

Analyte	Concn, µg/L[b]	Rec., %	Reagent water		Finished drinking water		Reagent water		Finished drinking water	
			s_r	s_R	s_r	s_R	RSD_r	RSD_R	RSD_r	RSD_R
Atrazine dealkylated	5.0	89.6	0.43	0.82	0.48	0.75	9.8	18.3	11.1	17.2
Barban	10.0	88.4	1.06	1.31	0.77	1.15	12.0	14.8	8.5	12.5
Carbofuran phenol	50.0	75.0	5.25	8.0	5.16	13.4	14.0	21.2	14.7	38.4
Cyanazine	10.0	91.6	1.13	1.74	0.85	1.29	12.4	19.0	9.3	14.0
Diuron	1.0	102	0.07	0.13	0.05	0.11	7.0	13.3	5.5	11.2
Fenamiphos sulfone	100.0	96.4	8.00	11.7	7.00	10.9	8.3	12.1	7.3	11.2
Fenamiphos sulfoxide	20.0	97.5	1.11	2.19	1.34	1.30	5.7	11.2	7.5	7.2
Fluometuron	2.0	88.0	0.21	0.29	0.21	0.31	11.9	17.0	11.7	17.0
3-Ketocarbofuran phenol	5.0	87.6	0.71	0.83	0.73	0.93	16.2	19.0	18.7	23.8
Linuron	2.0	94.5	0.11	0.14	0.12	0.24	6.2	7.8	6.6	12.9
Metribuzin DA	5.0	81.2	0.66	0.66	0.36	1.24	16.4	16.4	11.2	38.6
Metribuzin DADK	5.0	23.4	0.19	0.20	0.12	0.25	16.4	17.2	14.3	29.3
Metribuzin DK	5.0	39.0	0.21	0.34	0.31	0.58	10.6	17.5	15.5	28.8
Neburon	2.0	90.5	0.17	0.20	0.12	0.24	9.7	11.0	7.1	13.3
Pronamide metabolite	10.0	103	1.01	1.49	0.86	1.74	9.8	14.5	8.6	17.3
Propanil	1.0	98.0	0.08	0.11	0.08	0.12	8.5	11.5	8.7	13.8
Propham	10.0	86.3	1.52	2.08	0.62	1.09	17.6	24.1	7.3	12.8
Swep	10.0	95.1	0.54	0.81	0.39	0.54	5.7	8.6	4.0	5.5
Av.							11.0	15.2	9.9	18.0

[a] s_r and s_R = standard deviations for repeatability and reproducibility, respectively. RSD_r and RSD_R = corresponding relative standard deviations.
[b] Concentration value is approximately 15 times estimated MDL.

(**l**) *Separatory funnel shaker.*—Capable of shaking 2 L separatory funnels with rocking motion to thoroughly mix funnel contents (Eberbach Co., Ann Arbor, MI is suitable).

(**m**) *Tumbler.*—Capable of turning tumbler bottles end-over-end at 30 rpm (Associated Design and Mfg. Co., Alexandria, VA is suitable).

(**n**) *Boiling stones.*—Carborundum, No. 12 granules. Heat 30 min at 400°C before use. Cool and store in dessicator.

(**o**) *Water bath.*—Capable of maintaining 65–70 ± 2°F. Use in hood.

(**p**) *Analytical balance.*—Capable of weighing to nearest 0.0001 g.

(**q**) *Macrofilters.*—For water phases; 47 mm diam., 0.45 µm non-gridded, cellulose acetate filters: for organic phases; 47 mm diam., 0.5 µm non-gridded, PTFE filters.

(**r**) *Microfiltration apparatus.*—Syringe, (**k**) fitted with polypropylene filter holder with female Luer-Lock inlet and male Luer outlet. Use 0.45 µm, 0.2 cm^2 PTFE filters (Gelman LC 3S is suitable).

(**s**) *LC system.*—With high pressure syringes or sample injection loop capable of injecting 10–100 µL, analytical columns, gradient pumping system capable of constant flow, UV detector capable of monitoring at 254 nm, and strip chart recorder. Data system is recommended for measuring peak areas. Primary column: 250 mm × 4.6 mm id stainless steel packed with 5 µm ODS (Dupont Zorbax is suitable). Operating conditions: Linear gradient from acetonitrile–water (25 + 75), each with 0.1% phosphoric acid, to acetonitrile–water (90 + 10) in 25 min; hold 10 min; ramp to original phase composition, and equilibrate 15 min; flow rate, 1.0 mL/min; injection volume, 10 µL. Confirmation column: 250 mm × 4.6 mm id stainless steel packed with 5 µm silica (Dupont Zorbax Silica is suitable). Operating conditions: Hold 3 min at 5% methanol in CH_2Cl_2–hexane (5 + 95); linear gradient to 5% methanol in CH_2Cl_2–hexane (85 + 15) in 25 min; ramp to original phase composition, and equilibrate 20 min; flow rate, 1.0 mL/min; injection volume, 20 µL. Endcapped columns or different equipment configurations may require adjustments to operating conditions to maximize resolution.

C. Reagents

(**a**) *Standard solutions.*—Use standards of test compounds with purity >96% to prepare stock solutions at 1 mg/mL in methanol. Commercially prepared stock standards may be used at any concentration if certified by manufacturer or independent source. Store solutions at room temperature in TFE-fluorocarbon-sealed screw cap vials and protect from light. Replace stock solutions after 2 months, or sooner if comparison with laboratory control standards indicates degradation.

(**b**) *Internal standard solution.*—2.0 mg/mL of ethylbenzene (stock solution purity >96%) in methanol.

(**c**) *Surrogate solution.*—0.1 mg/mL of carbazole in methanol. Store surrogate stock solution in TFE-fluorocarbon-sealed screw cap bottle at room temperature.

(**d**) *Instrument QC standard stock solutions.*—Dissolve 0.0010 g of each: carbazole, neburon, ethylbenzene, fenami-

phos sulfoxide, and fluometuron in methanol in separate 10 mL volumetric flasks. Dilute to volume. Combine 100 µL carbazole stock solution, 5 mL ethylbenzene stock solution, 800 µL fenamiphos sulfoxide stock solution, 100 µL neburon stock solution, and 20 µL fluometuron stock solution in 100 mL volumetric flask and dilute to volume with methanol.

(**e**) *Solvents.*—Acetone, methylene chloride, hexane, methanol, water, distilled-in-glass quality or equivalent.

(**f**) *Phosphate buffer, pH 7.*—Mix 29.6 mL 0.1N HCl and 50 mL 0.1M dipotassium hydrogen phosphate (K_2HPO_4).

(**g**) *Phosphoric acid.*—85.1% H_3PO_4 assay.

(**h**) *Sodium sulfate.*—Granular, anhydrous. Heat 4 h in shallow tray at 450°C.

(**i**) *Sodium chloride.*—Crystals. Heat 4 h in shallow tray at 450°C.

(**j**) *Ethylbenzene.*—Purity >98%.

(**k**) *Carbazole.*—Purity >98%.

(**l**) *Reagent water.*—Water determined to be free of interfering contaminants.

(**m**) *Preservative.*—Mercuric chloride solution. 10 mg $HgCl_2$/mL in reagent water, (**l**). *Caution*: Mercuric chloride is highly toxic and a suspected carcinogen. Wear appropriate safety equipment to prevent inhalation or skin absorption.

D. Sample Collection

Add 1 mL preservative, **C(m)**, to glass sample bottles, **B(a)** (not prerinsed with sample), and collect 1 L grab sample by conventional sampling practices. Seal bottles and shake vigorously 1 min. Refrigerate samples at 4°C until extraction. Extract samples within 28 days.

E. Sample Preparation

(**a**) *Automated extraction method.*—Add preservative, **C(m)**, to samples not previously preserved as in **D**. Mark water meniscus on side of grab sample bottle for later determination of sample volume. Add 50 µL surrogate solution, **C(c)**, to 1 L grab sample prior to extraction (to produce 5.0 µg/L surrogate concentration in sample, 1.0 µg/mL in extract). Pour entire sample into 2 L separatory funnel (if mechanical separatory funnel shaker is used) or into tumbler bottle (if mechanical tumbler is used). Add 50 mL phosphate buffer, **C(f)**. Check pH of sample solution with pH paper, and adjust to pH 7 by adding H_2SO_4 or NaOH if necessary. Add 100 g NaCl to sample solution, seal, and shake to dissolve.

Add 300 mL CH_2Cl_2 to emptied grab sample bottle, seal, and shake 30 s to rinse inner walls. Transfer rinse to sample solution in separatory funnel or tumbler bottle, seal, and shake sample 10 s, with periodic venting. Repeat shaking and venting until pressure release is not observed. Reseal and place sample container in appropriate mechanical mixing device (separatory funnel shaker or tumbler). Shake or tumble sample 1 h. Complete and thorough mixing of organic and aqueous phases should be observed in 2 min. After extraction, pour contents of tumbler bottle into 2 L separatory funnel. Allow 10 min for layers to separate. If emulsion interface between layers is more than one-third volume of solvent layer, complete phase separation by mechanical techniques, such as stirring, filtration of emulsion through glass wool or centrifugation. Collect CH_2Cl_2 extract (lower layer) in 500 mL Erlenmeyer flask containing ca 5 g anhydrous Na_2SO_4, **C(h)**. Swirl flask to dry extract; let flask sit 15 min.

Determine original sample volume by refilling sample bottle to mark with water then transferring water to 1000 mL graduated cylinder. Record sample volume to nearest 5 mL.

(**b**) *Manual extraction method.*—Add preservative, **C(m)**, to samples not previously preserved as in **D**. Mark water meniscus on side of grab sample bottle for later determination of sample volume. Pour entire sample into 2 L separatory funnel and add 50 µL surrogate solution, **C(c)**, to 1 L sample prior to extraction (to produce 5.0 µg/L surrogate concentration in sample, 1.0 µg/mL in extract). Add 50 mL phosphate buffer, **C(f)**. Check pH of sample solution with pH paper, and adjust to pH 7 by adding H_2SO_4 or NaOH if necessary. Add 100 g NaCl to sample solution, seal, and shake to dissolve.

Add 60 mL CH_2Cl_2 to emptied grab sample bottle, seal, and shake 30 s to rinse inner walls. Transfer rinse to sample solution in separatory funnel. Extract sample by vigorously shaking funnel 2 min with periodic venting to release excess pressure. Allow 10 min for layers to separate. If emulsion interface between layers is more than one-third volume of solvent layer, complete phase separation by mechanical techniques, such as stirring, filtration of emulsion through glass wool or centrifugation. Collect CH_2Cl_2 extract (lower layer) in 500 mL Erlenmeyer flask containing ca 5 g anhydrous Na_2SO_4. Add second 60 mL of CH_2Cl_2 to emptied grab sample bottle and repeat rinsing, transferring, and extracting procedure, using same separatory funnel and combining extracts in same Erlenmeyer flask. Repeat entire procedure a third time with 60 mL CH_2Cl_2. Swirl flask to dry extract; let flask sit for 15 min.

Determine original sample volume as in **E(a)**.

F. Extract Concentration

Assemble K-D concentrator by attaching 25 mL concentrator tube to 500 mL evaporation flask. Pass combined extract through drying column containing ca 10 cm anhydrous Na_2SO_4, **C(h)**, and collect extract in K-D concentrator. Rinse Erlenmeyer flask and column with 20–30 mL CH_2Cl_2 to complete quantitative transfer.

Add 1 or 2 clean boiling stones to evaporation flask and attach macro-Snyder column. Prewet top of column by adding ca 1 mL CH_2Cl_2. Place K-D apparatus on 65–70°F water bath so concentrator tube is partially immersed in hot water, and entire lower rounded surface of flask is bathed with hot vapor. Adjust vertical position of apparatus and water temperature to complete concentration process in 15–20 min. At proper rate of distillation, column balls will actively chatter, but chambers will not flood. When volume of liquid is ca 2 mL, remove K-D apparatus from water bath, drain column, and cool ≥10 min.

Remove macro-Snyder column; rinse flask and lower joint with 1–2 mL methanol into concentrator tube. Add 5–10 mL methanol and fresh boiling stone. Attach micro-Snyder column to concentrator tube and prewet top of column by adding ca 0.5 mL methanol. Place micro K-D apparatus on water bath so

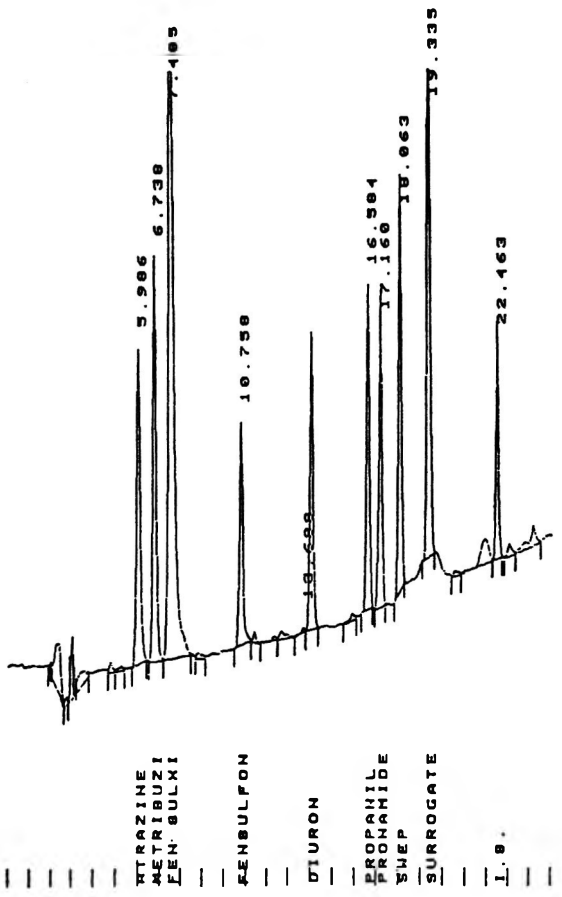

Figure 992.14A. LC Chromatogram of group A compounds analyzed on Dupont Zorbax column. Operating conditions: linear gradient from 25 + 75 acetonitrile (0.1% H_3PO_4)–water (5% acetonitrile, 0.1% H_3PO_4) to 90 + 10 acetonitrile–water in 25 min; flow, 1.0 mL/min; injection, 10.0 μL; wavelength, 254 nm.

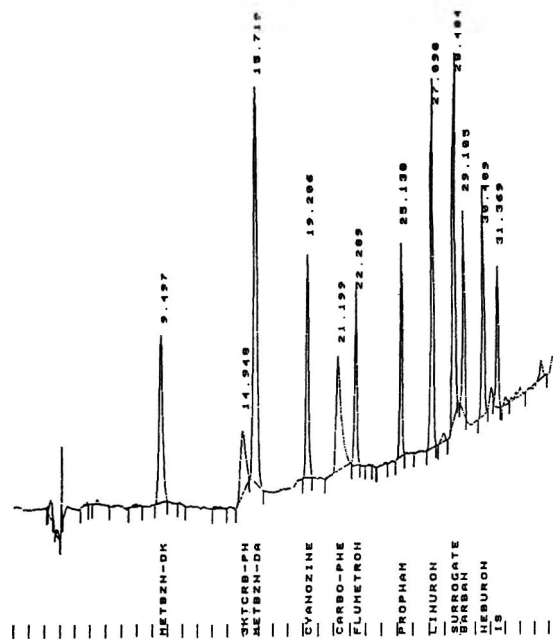

Figure 992.14B. Liquid chromatogram of group B compounds analyzed on Dupont Zorbax column. Operating conditions: Hold 8 min at 20 + 80 acetonitrile (0.1% H_3PO_4)–water (5% acetonitrile, 0.1% H_3PO_4), then linear gradient to 90 + 10 acetonitrile–water in 25 min, hold 15 min; flow, 1.0 mL/min; injection, 10.0 μL; wavelength, 254 nm.

concentrator tube is partially immersed in hot water. Adjust vertical position of apparatus and water temperature to complete concentration process in 5–10 min. When volume of liquid is ca 2 mL, remove apparatus from bath, let column drain, and cool. Remove micro-Snyder column, and rinse walls of concentrator tube while adjusting volume to 5.0 mL with methanol. Add 50 μL internal standard stock solution, **C(b)**, to sample extract (final internal standard concentration of 20 μg/mL), seal, and shake. Transfer extract to appropriate sized TFE-fluorocarbon-sealed screw cap vial and store at 4°C until analysis. Extracts are stable under these conditions for at least 28 days.

G. Calibration of Liquid Chromatograph with UV Detector

Examples of separations using method conditions are shown in Figures **992.14A** and **992.14B**. Initially, perform 5-level calibration within linear range of UV detector (254 nm), using internal standard and relative response factors. If response factor (RF) value over working range is constant (≤10% RSD), average RF can be used for calculations. Verify calibration curve daily using 1 or 2 calibration standards. If response of any analyte varies >20% from average relative response factor for initial calibration, analysis of single-level standard must be repeated with fresh standard. Alternatively, new calibration curve can be prepared.

H. Quality Control

Minimum quality control requirements include: (*1*) initial demonstration of laboratory capability, (*2*) analysis of surrogate standards as continuing check on sample preparation (acceptable recovery is 70–130%), (*3*) monitoring of internal standard area counts as continuing check of system performance (area of internal standard should be ≤30% of average area in calibration standards), (*4*) analysis of method blank with each set of extracted samples as continuing check on sample contamination, (*5*) analysis of spiked reagent water as continuing check on method recovery, (*6*) analysis of daily instrument QC standard to ensure acceptable instrument performance (Table **992.14B**), and (*7*) analysis of an external unknown performance evaluation sample (when available) at least once a year.

Demonstrate initial method performance by extracting four 1 L samples of spiked reagent water at concentration levels indicated in Table **992.14C** (ca 15× estimated method detection limit). Calculate average percent recovery and standard deviation from results of these 4 analyses. For acceptable performance, percent relative standard deviation should be <20%, and analyte mean recoveries should be within acceptance lim-

Table 992.14B. Instrument quality control standard

Test	Analyte	Concn, µg/mL	Requirements
Sensitivity	Fluometuron	0.02	Detection of analyte; S/N >3
Chromatographic performance	Fenamiphos sulfoxide	0.8	0.95<PSF<1.05[a] 0.93<PGF<1.07[b]
Column performance	Neburon	0.1	Resolution >0.5[c]
	Ethylbenzene (IS)	5	—

[a] PSF = peak symmetry factor. Calculated as follows: PSF = $w^{1/2}/(0.5 \times W^{1/2})$ where $w^{1/2}$ is width of peak front assuming peak is split at its highest point at half weight and $W^{1/2}$ is peak width at half height.
[b] PGF = peak Gaussian factor. Calculated as follows: PGF = $(1.83 \times W^{1/2})/W^{1/10}$ where $W^{1/2}$ is peak width at half height and $W^{1/10}$ is peak width at tenth height.
[c] Resolution between the 2 peaks as defined by the equation: R = t/W where t is difference in elution times between the 2 peaks and W is average peak width, at the base line, of the 2 peaks.

its presented in Table **992.14C**. Check method recovery on continuing basis by analyzing 1 reagent water sample, spiked at concentration levels indicated in Table **992.14C**, with each sample set extracted within 24 h period. Compare recovery to performance-based acceptance limits in Table **992.14C**.

Ref.: JAOAC **75**, September/October issue (1992)
CAS-101-27-9 (barban)
CAS-1563-38-8 (carbofuran phenol)
CAS-21725-46-2 (cyanazine)
CAS-330-54-1 (diuron)
CAS-31972-44-8 (fenamiphos sulfone)
CAS-31972-43-7 (fenamiphos sulfoxide)
CAS-2164-17-2 (fluometuron)
CAS-17781-16-7 (3-ketocarbofuran)
CAS-330-55-2 (linuron)
CAS-35045-02-4 (metribuzin DA)
CAS-36507-37-0 (metribuzin DK)
CAS-55-53-7-3 (neburon)
CAS-709-98-8 (propanil)
CAS-122-42-9 (propham)
CAS-1918-18-9 (swep)

Results and Discussion

Treatment of Data

The returned data, from 10 laboratories, were grouped by water type, were arranged into 6 subsets by sample, and were evaluated analyte by analyte using the IMVS computer program. First, missing data points were replaced by values esti-

Table 992.14C. Acceptance limits (as percent of spike value) for analysis of instrument quality control standard

Analyte	Concn level[a]	Mean rec., %[b]	Overall SD[b]	Acceptance limits,%[c]
Atrazine dealkylated	5.0	4.48	0.82	40–139
Barban	10.0	8.84	1.31	49–128
Carbofuran phenol	50.0	37.5	8.00	27–123
Cyanazine	10.0	9.16	1.74	39–144
Diuron	1.0	1.02	0.13	63–141
Fenamiphos sulfone	100	96.4	11.7	61–132
Fenamiphos sulfoxide	20.0	19.5	2.19	65–130
Fluometuron	2.0	1.76	0.29	45–132
3-Ketocarbofuran phenol	5.0	4.38	0.83	38–137
Linuron	2.0	1.89	0.14	74–116
Metribuzin DA	5.0	4.06	0.66	42–121
Metribuzin DADK	5.0	1.17	0.20	11–35
Metribuzin DK	5.0	1.95	0.34	19–59
Neburon	2.0	1.81	0.20	61–121
Pronamide metabolite	10.0	10.3	1.49	58–148
Propanil	1.0	0.98	0.11	65–131
Propham	10.0	8.63	2.08	24–149
Swep	10.0	9.51	0.81	71–119

[a] Concn level approximately 15 times estimated MDL, µg/L.
[b] Calculated from the regression equations for mean recovery and overall standard deviation for reagent water matrix reported in the collaborative study, µg/L.
[c] Acceptance limits are defined as the (mean recovery ±3 standard deviations)/true value.

mated by interpolation (2), and "less than" and "nondetect" values were converted to zero. Then outlier tests suggested in ASTM Standard Practice D2777-86 (4) were applied. The first outlier test was Youden's laboratory ranking procedure, which rejected laboratories that had a consistently higher or lower bias in their submitted data for a given analyte compared to the other laboratories. If a bias was determined, all 6 analyte values for that laboratory were rejected. This procedure was applied to each analyte data set, for each water type, at the 5% level of significance. Next, zeroes and interpolated values were removed before further evaluation. As a final outlier test, Thompson's test for individual outliers (5) was applied to the data using a 5% significance level. If an individual data point was rejected based on this test, it was removed from the subset, and the test was repeated once more.

Summary statistics were calculated for mean recovery and overall method precision for each of the 6 concentration levels. Single-analyst precision was calculated for each of the 3 concentration pairs. The IMVS program used these summary statistics to calculate relationships between mean recovery and true concentration and between precision and mean recovery in the form of linear regression equations, using the weighted least-squares technique (6, 7). Coefficients of determination of the weighted regression equations (COD_w) were also calculated to evaluate the fit of the retained data sets. High variability in the precision estimates at different concentrations in some data sets resulted in very low or negative COD_w values. However, the regression equations presented in Table 1 can be used to estimate the mean method recovery, overall precision, and single-analyst precision at any value within the concentration range studied. IMVS also determined statistically significant matrix effects between water types for each of the 18 analytes.

Quality control (QC) requirements were incorporated into this study to monitor the "in control" status of the various stages of the analytical process. Each laboratory was instructed to extract a QC sample, containing 8 analytes ranging in concentration from 1.2 to 12.4 μg/L, for analysis after both the reagent water matrix set and the drinking water matrix set, and then to evaluate their QC recovery results vs acceptance limits of 70–130%. Four laboratories reported all QC analyses within acceptance limits, 4 laboratories reported only 1 analyte beyond the acceptance limits, 1 laboratory reported 2 analytes beyond the acceptance limits, and a final laboratory reported 4 analytes beyond the acceptance limits. Of the 10 QC data points beyond acceptance limits, 6 resulted from the poor recovery of 3-ketocarbofuran phenol. This compound and metribuzin DA suffered from poor peak geometry and poor resolution. The 2 laboratories that produced the most QC data outside of acceptance limits also had the most data rejected, which supports the applicability of QC samples to assess overall data quality. Nevertheless, no data points were removed from the study data sets before outlier testing based on these QC sample results.

The column recommended in this method has undergone an endcapping process designed to further deactivate silanol sites on the silica support. The extent of this endcapping process greatly affects column performance and, therefore, gradient conditions. The gradient conditions specified in the method could not separate all 18 analytes from a single solution, so analytes were divided into 2 solutions to assure peak resolution (Table 2). Future improvements in the endcapping process or support media may improve gradient conditions and allow resolution of the analytes from a single solution.

The number of false negatives was evaluated to determine the usefulness of the method at the 2 lowest concentration levels, approximately 5–10× the estimated method detection limits (Table 2). At the lowest concentration level there were 28 occurrences of reported zeros for 360 submitted data points (180 data per water type). Four compounds (carbofuran phenol, 3-ketocarbofuran phenol, metribuzin DADK, and metribuzin DK) accounted for 17 of these occurrences. Laboratories 2 and 15 accounted for 22 of the 28 false negatives reported. At the next higher concentration level there were only 14 reported false negatives for 360 submitted data points, with the same 4 compounds accounting for 12 of the occurrences. Laboratories 2 and 15 accounted for 9 of the 14 false negatives.

The false negatives reported for carbofuran phenol and 3-ketocarbofuran phenol may have resulted from their low sensitivity and tailing peaks, coupled with poor resolution from the next eluting compound. Carbofuran phenol eluted just before fluometuron, while 3-ketocarbofuran phenol eluted just before metribuzin DA. Depending on the resolution between these elution pairs, the tailing peaks would be more or less obscured by the onset of the next peak, resulting in poor area integrations. On the other hand, the study design concentrations did not properly address the poor extraction efficiency (ca 18 and 41%, respectively) of metribuzin DADK and metribuzin DK, and as a result, their concentration levels of their extracts may have been near or below the method detection limit for some of the collaborator's laboratories.

Laboratory 15 reported using a Hypersil column instead of the Zorbax column recommended in the method. Although this column produced a large number of false negatives at the 2 lowest concentration levels, it did not produce an unusually large number of rejected data for the remainder of the data set.

Laboratory 2 used the specified Zorbax column and initial mobile phase composition of 25 + 75 (acetonitrile–water), but ramped to a final composition of only 60 + 40 in 3 gradient steps, instead of the gradient from 25 + 75 to 90 + 10 specified in the method. Not achieving a sufficiently high organic phase may have contributed to poor resolution of the 4 compounds listed above.

A rising base line at the high attenuation settings required to measure the signal produced from a 10 μL injection was a persistent problem reported by nearly all the laboratories. Only 4 laboratories were able to complete the study using a 10 μL injection loop. The other laboratories used longer injection loops (from 25 μL to 100 μL) to analyze the samples at less sensitive attenuation settings. No relationship was found between injection volume and the number of outliers for each laboratory.

Rejection of Outliers

Overall, the IMVS program rejected 20.9% (452) of the 2160 data points submitted as outliers: 19.2% for the reagent

Table 1. Summary statistics and regression equations for EPA Method 4 collaborative study data sets (μg/L)

	Reagent water						Drinking water				
Analyte	C[a]	N	X[b]	s_R[c]	s_r[d]	Regression equation	N	X	s_R	s_r	Regression equation
Atrazine dealkylated	2.00	7	1.70	0.36	0.28		7	1.72	0.42	0.26	
	3.00	7	2.92	0.36	—		8	2.58	0.40	—	
	12.00	8	11.22	2.15	1.06		9	10.68	1.29	0.89	
	15.00	8	13.04	2.69	—		9	13.40	2.40	—	
	30.00	8	26.76	4.60	2.14		9	25.71	5.38	4.34	
	40.00	8	34.79	6.70	—		8	35.85	3.55	—	
X						0.897C + 0.005					0.886C − 0.058
s_R						0.184X − 0.004					0.140X + 0.140
s_r						0.071X + 0.121					0.108X + 0.017
Barban	4.21	9	3.32	0.91	0.56		6	3.85	0.91	0.59	
	6.31	9	5.48	0.97	—		8	5.61	0.92	—	
	25.20	9	22.66	3.24	4.44		8	22.76	1.37	1.75	
	31.50	9	28.28	3.83	—		8	29.59	2.31	—	
	63.10	8	60.61	1.87	3.63		8	56.09	5.40	2.86	
	84.10	9	80.30	5.88	—		8	80.55	3.72	—	
X						0.947C − 0.632					0.923C − 0.093
s_R						0.075X + 0.650[e]					0.051X + 0.685
s_r						2.88[f]					0.043X + 0.386
Carbofuran phenol	20.00	7	14.99	2.90	1.98		5	17.10	5.02	3.68	
	30.00	8	19.97	2.56	—		5	20.60	4.65	—	
	120.0	9	81.11	28.13	22.1		7	67.43	42.04	6.92	
	150.0	9	109.4	38.39	—		7	93.54	47.33	—	
	300.0	8	255.9	29.78	23.8		7	197.4	91.11	33.7	
	400.0	9	339.2	52.32	—		7	304.0	94.53	—	
X						0.784C − 1.740					0.632C + 3.420
s_R						0.238X − 0.938[e]					0.492X − 3.780
s_r						15.96[f]					0.099X + 1.700
Cyanazine	2.20	10	1.99	1.05	0.53		8	2.25	0.44	0.33	
	3.30	10	2.54	1.24	—		8	3.15	0.67	—	
	13.20	10	12.65	2.29	2.38		7	12.21	0.84	1.03	
	16.50	10	15.09	2.98	—		8	14.66	1.98	—	
	33.00	10	31.09	2.58	1.47		7	28.23	3.30	3.42	
	44.00	9	41.11	3.30	—		8	42.04	7.68	—	
X						0.937C − 0.211					0.893C + 0.262
s_R						0.089X + 0.930[e]					0.120X + 0.193
s_r						1.46[f]					0.083X + 0.096
Diuron	0.25	8	0.29	0.10	0.04		8	0.30	0.06	0.03	
	0.38	9	0.45	0.10	—		8	0.41	0.09	—	
	1.50	9	1.50	0.15	0.13		8	1.48	0.13	0.07	
	1.87	9	1.78	0.15	—		7	1.88	0.13	—	
	3.75	9	3.56	0.31	0.18		7	3.68	0.31	0.24	
	5.00	8	4.84	0.38	—		8	4.83	0.42	—	
X						0.946C + 0.068					0.960C + 0.056
s_R						0.054X + 0.081					0.065X + 0.048
s_r						0.052X + 0.018					0.045X + 0.010

Table 1. ***Continued***

	Reagent water						Drinking water				
Analyte	C*a*	N	X[b]	s_R[c]	s_r[d]	Regression equation	N	X	s_R	s_r	Regression equation
Fenamiphos sulfone	30.00	9	32.79	4.82	4.65		7	31.29	5.91	5.62	
	37.50	10	37.17	5.52	—		7	34.79	4.64	—	
	90.00	10	87.83	10.00	8.72		7	91.07	5.60	8.53	
	112.0	10	105.5	15.73	—		7	109.0	11.58	—	
	225.0	10	208.6	29.67	14.76		7	219.6	33.01	7.61	
	300.0	10	290.3	19.85	—		6	277.7	25.64	—	
X						0.923C + 4.100					0.957C + 1.400
s_R						0.106X + 1.540					0.090X + 2.180
s_r						0.053X + 2.860					7.25[f]
Fenamiphos sulfoxide	22.40	7	22.20	3.12	1.45		6	20.65	1.32	1.46	
	28.00	7	25.99	2.03	—		7	25.49	2.10	—	
	67.30	8	65.54	8.19	5.75		7	65.60	4.31	1.93	
	84.10	8	79.59	8.48	—		7	83.04	6.15	—	
	168.0	8	160.7	13.45	13.08		7	168.4	17.70	5.04	
	224.0	8	206.0	28.63	—		6	214.3	8.16	—	
X						0.939C + 0.691					0.995C – 1.860
s_R						0.111X + 0.037					0.071X + 0.024
s_r						0.078X – 0.404					0.017X + 1.040
Fluometuron	0.50	7	0.39	0.06	0.09		10	0.53	0.19	0.12	
	0.75	9	0.62	0.25	—		10	0.69	0.12	—	
	3.00	8	2.58	0.44	0.48		10	2.76	0.43	0.33	
	3.75	9	3.42	0.54	—		10	3.34	0.34	—	
	7.50	9	6.78	0.66	0.46		9	6.33	0.78	0.65	
	10.00	9	9.21	1.12	—		10	9.21	1.78	—	
X						0.914C – 0.066					0.872C + 0.081
s_R						0.144X + 0.046					0.118X + 0.096
s_r						0.34[f]					0.079X + 0.071
3-Ketocarbofuran phenol	1.30	9	1.21	0.21	0.35		6	1.07	0.41	0.29	
	1.95	8	1.64	0.48	—		5	1.51	0.20	—	
	7.80	9	6.62	1.43	1.30		7	5.43	1.16	0.98	
	9.75	9	8.38	1.41	—		7	7.74	1.25	—	
	19.50	9	17.06	2.04	2.14		7	15.13	3.62	3.87	
	26.00	9	23.86	4.78	—		7	22.79	7.66	—	
X						0.870C + 0.032					0.785C + 0.017
s_R						0.175X + 0.069					0.223X + 0.060
s_r						0.121X + 0.182					0.173X + 0.057
Linuron	0.50	7	0.45	0.08	0.08		7	0.57	0.14	0.07	
	0.75	8	0.70	0.13	—		8	0.78	0.24	—	
	3.00	7	2.82	0.10	0.08		7	2.69	0.28	0.21	
	3.75	6	3.63	0.09	—		8	3.56	0.23	—	
	7.50	8	7.16	0.41	0.48		7	6.62	0.67	0.38	
	10.00	8	9.58	0.80	—		7	9.52	0.51	—	
X						0.962C – 0.031					0.895C + 0.117
s_R						0.041X + 0.070					0.056X + 0.140
s_r						0.030X + 0.060					0.048X + 0.035

Table 1. ***Continued***

		Reagent water					Drinking water				
Analyte	C[a]	N	X[b]	s_R[c]	s_r[d]	Regression equation	N	X	s_R	s_r	Regression equation
Metribuzin DA	1.00	8	0.73	0.13	0.24		8	0.72	0.32	0.15	
	1.50	8	1.08	0.32	—		8	0.99	0.46	—	
	6.00	9	4.85	0.64	1.29		8	3.53	1.56	0.37	
	7.50	9	5.77	1.61	—		8	4.80	1.79	—	
	15.00	9	12.59	1.10	1.01		8	9.50	3.29	1.31	
	20.00	9	17.64	1.77	—		8	13.75	4.69	—	
X						0.837C − 0.130					0.630C + 0.069
s_R						0.147X + 0.069[e]					0.361X + 0.081
s_r						0.85[f]					0.090X + 0.073
Metribuzin DADK	0.85	3	0.42	0.24	0.22		6	0.20	0.15	0.07	
	1.27	4	0.30	0.07	—		5	0.24	0.04	—	
	5.09	6	1.18	0.22	0.12		6	0.85	0.21	0.05	
	6.36	5	1.47	0.06	—		6	1.03	0.25	—	
	12.70	6	2.91	0.49	0.24		6	2.10	0.62	0.46	
	17.00	5	3.83	0.28	—		6	3.09	1.05	—	
X						0.195C + 0.198					0.162C + 0.051
s_R						0.089X + 0.097[e]					0.228X + 0.056
s_r						0.19[f]					0.092X + 0.044
Metribuzin DK	1.00	6	0.28	0.05	0.11		6	0.34	0.06	0.22	
	1.50	7	0.52	0.16	—		8	0.66	0.37	—	
	6.00	8	2.43	0.43	0.32		8	2.40	0.73	0.37	
	7.50	8	2.86	0.38	—		8	2.98	0.81	—	
	15.00	8	6.16	0.96	0.32		8	5.71	1.48	0.57	
	20.00	7	8.16	1.32	—		8	8.59	1.49	—	
X						0.416C − 0.128					0.411C − 0.040
s_R						0.165X + 0.020					0.284X + 0.008[e]
s_r						0.25[f]					0.058X + 0.195
Neburon	0.30	7	0.26	0.04	0.03		10	0.28	0.07	0.03	
	0.45	8	0.38	0.05	—		10	0.43	0.08	—	
	1.80	8	1.56	0.26	0.30		10	1.60	0.20	0.14	
	2.25	8	1.96	0.32	—		10	2.05	0.18	—	
	4.50	8	4.15	0.27	0.16		10	3.99	0.64	0.30	
	6.00	7	5.91	0.28	—		10	5.58	0.77	—	
X						0.921C − 0.027					0.902C + 0.014
s_R						0.20[f]					0.113X + 0.037
s_r						0.16[f]					0.068X + 0.005
Pronamide metabolite	2.47	7	2.87	0.96	0.64		9	2.71	1.20	0.54	
	3.71	8	4.60	1.18	—		9	4.10	0.82	—	
	14.80	8	14.86	1.95	1.65		9	14.69	1.67	1.19	
	18.60	8	17.91	2.12	—		9	18.53	3.16	—	
	37.10	8	36.10	2.86	2.17		9	35.48	6.21	2.48	
	49.50	8	48.27	3.64	—		8	47.52	4.20	—	
X						0.961C + 0.655					0.963C + 0.395
s_R						0.068X + 0.800					0.095X + 0.790
s_r						0.056X + 0.436					0.050X + 0.366

Table 1. ***Continued***

	Reagent water						Drinking water				
Analyte	Ca	N	X[b]	s_R[c]	s_r[d]	Regression equation	N	X	s_R	s_r	Regression equation
Propanil	0.25	8	0.26	0.07	0.05		7	0.20	0.05	0.05	
	0.38	7	0.40	0.02	—		7	0.36	0.04	—	
	1.50	9	1.50	0.20	0.13		8	1.43	0.15	0.09	
	1.87	9	1.75	0.21	—		8	1.76	0.27		
	3.75	9	3.55	0.37	0.17		8	3.45	0.56	0.25	
	5.00	8	4.79	0.36	—		8	4.82	0.50	—	
X						0.949C + 0.031					0.965 – 0.031
s_R						0.081X + 0.034					0.112X + 0.025
s_r						0.043X + 0.041					0.043X + 0.041
Propham	4.40	9	3.37	1.21	1.01		8	3.62	0.62	0.40	
	6.60	10	4.98	1.45	—		8	5.54	0.80	—	
	26.40	10	26.24	8.72	5.43		9	22.11	2.03	1.74	
	33.00	9	28.88	4.89	—		9	28.88	3.73	—	
	66.00	8	62.37	3.68	4.15		9	55.37	7.94	3.54	
	88.00	10	85.61	14.74	—		8	82.20	4.61	—	
X						0.965C – 1.020					0.879C – 0.262
s_R						0.163X + 0.675					0.098X + 0.264
s_r						3.53[f]					0.056X + 0.146
Swep	0.52	7	0.47	0.12	0.10		10	0.55	0.16	0.09	
	0.78	7	0.80	0.05	—		10	0.82	0.13	—	
	3.13	8	3.02	0.43	0.26		10	3.13	0.17	0.14	
	3.92	8	3.59	0.37	—		10	3.90	0.27	—	
	7.83	8	7.27	0.74	0.45		10	7.84	0.73	0.48	
	10.40	7	10.04	0.66	—		10	10.33	0.61	—	
X						0.951C – 0.003					0.990C + 0.039
s_R						0.079X + 0.062					0.043X + 0.118
s_r						0.050X + 0.071					0.033X + 0.069

[a] Spike concn, µg/L.
[b] Mean recovery, µg/L.
[c] Overall standard deviation, µg/L.
[d] Single-analyst standard deviation, µg/L.
[e] COD_w <0.50. Use of regression equation outside of study range is not recommended.
[f] COD_w ≤0.00 for weighted regression equation. Average precision is reported.

water data set and 22.6% for the finished drinking water data set (Figure 1). Metribuzin DADK, fenamiphos sulfoxide, carbofuran phenol, linuron, metribuzin DK, fenamiphos sulfone, 3-ketocarbofuran phenol, and swep lost more than 22.2% of their data points as outliers, exceeding the 2/9 rule recommended by AOAC (8).

The laboratory ranking test accounted for 78.3% of all rejected data and detected a systematic high bias for Laboratory 9, rejecting 53.7% of its data as outliers, despite Laboratory 9 having reported no problems using the method (Figure 2).

Method Recovery

The summary statistics, after removal of outliers, are presented in Table 1. The COD_w calculated for the weighted linear regression equations for mean recovery were above 0.98 (except for carbofuran phenol in drinking water at 0.96), confirming the suitability of these equations for estimating mean recovery at all concentration levels within the range tested. COD_w are not reported here because they were used only as a screening tool for regression fits. Mean recoveries for 13 of the 18 pesticides, as estimated from the slopes of the regression equations, were greater than 85% in both sample matrixes. The carbofuran phenol (ca 72%), 3-ketocarbofuran phenol in drinking water (ca 78%), and metribuzin DA (ca 75%) recoveries were somewhat low; metribuzin DADK (ca 18%) and metribuzin DK (ca 41%) recoveries were poor. The poor recoveries for metribuzin DADK and metribuzin DK were consistent with the single-laboratory evaluation data (1), probably due to their poor extraction efficiency from water.

Table 2. Chemical Abstracts Service registry numbers, peak identification codes, and estimated method detection limits for 18 pesticides

Analyte	Chemical Abstracts Service registry No.	Identification code[a]	Estimated MDL, µg/L[b]
Atrazine dealkylated	—	A-1	0.25
Barban	101-27-9	B-9	0.50
Carbofuran phenol	1563-38-8	B-5	1.8
Cyanazine	21725-46-2	B-4	0.58
Diuron	330-54-1	A-5	0.070
Fenamiphos sulfone	31972-44-8	A-4	5.7
Fenamiphos sulfoxide	31972-43-7	A-3	1.0
Fluometuron	2164-17-2	B-6	0.10
3-Ketocarbofuran phenol	17781-16-7	B-2	0.25
Linuron	330-55-2	B-8	0.25
Metribuzin DA	35045-02-4	B-3	0.21
Metribuzin DADK	—	A-2	2.5
Metribuzin DK	36507-37-0	B-1	0.10
Neburon	555-37-3	B-10	0.15
Pronamide metabolite[c,d]	—	A-7	0.81
Propanil	709-98-8	A-6	0.067
Propham	122-42-9	B-7	0.75
Swep	1918-18-9	A-8	0.75

[a] Identification of peaks in Figures 992.14A and 992.14B. Letter indicates mix (A or B) containing analyte, IS = internal standard, and SUR = surrogate standard.

[b] Estimated method detection limit (MDL) was determined from results of 7 or more replicate analyses of a reagent water sample fortified with a concentration of analyte to yield a chromatographic peak with a 5 to 1 signal-to-noise ratio. The standard deviation of these data obtained using the method was multiplied by the student's *t* value appropriate for a 99% confidence level with n – 1 degrees of freedom. The MDL was defined as either the concentration of analyte yielding 5 to 1 signal-to-noise ratio or the calculated concentration, whichever was greater.

[c] *N*-(1,1-Dimethylacetonyl)-3,5-dichlorobenzamide.

[d] Rohm and Haas No. RH 24,580.

Precision

The overall standard deviation is the precision associated with measurements generated by a group of laboratories, while the single-analyst standard deviation is the precision associated with performance in an individual laboratory. The weighted linear regression equations in Table 1 describe method precision as a function of mean recovery. The COD_w calculated for these weighted equations show them to be representative of the submitted data sets. Experience has shown that a COD_w of <0.5 indicates that the study has failed to establish a definitive linear relationship in the retained data set. For overall precision, 7 of 36 regression equations had COD_w <0.50 (see note "e" Table 1). Of the 36 single-analyst precision regression equations, which were based on precision measurements at 3 mean concentration levels, 10 yielded a COD_w <0.50. Except for these equations with low COD_w, the equations can be used effectively to derive performance-based QC limits for this method.

The precision estimates calculated for a concentration value about 15× the estimated MDL using either the regression equations reported or the mean standard deviations observed are presented in Table **992.14A**. The average overall relative standard deviations at this concentration for all 18 pesticides in reagent water was 15.2%, and individually ranged from 7.8% for linuron to 24.1% for propham. In finished drinking water, the average RSD_R at this concentration was 18.0% and ranged from 5.5% for swep to 38.6% for metribuzin DA. The average single-analyst relative standard deviations for all 18 pesticides in reagent water was 11.0%, and individual estimates ranged from 5.7% for swep and fenamiphos sulfoxide to 17.6% for propham. In finished drinking water, the average RSD_r was 9.9%, and individual values ranged from 4.0% for swep to 18.7% for 3-ketocarbofuran phenol. The precision estimates improve at concentration levels above 15× the estimated MDL values used in these calculations.

Effect of Water Type

The data across water types were subjected to an analysis of variance test to determine the effect of water type on recovery and precision. The method exhibited statistically significant matrix effects due to water type for 3 compounds: carbofuran phenol, linuron, and metribuzin DADK. The method demonstrated clearly lower recovery and poorer precision for carbofuran phenol in finished drinking water samples than in reagent water. The linuron data, on the other hand, were so precise that recoveries in finished drinking water, which were only slightly lower than that found in reagent water, were statistically significant. The metribuzin DADK recoveries were so poor (<25% in both water types) that the statistical significance attributed to higher recoveries observed in reagent water were of little practical importance.

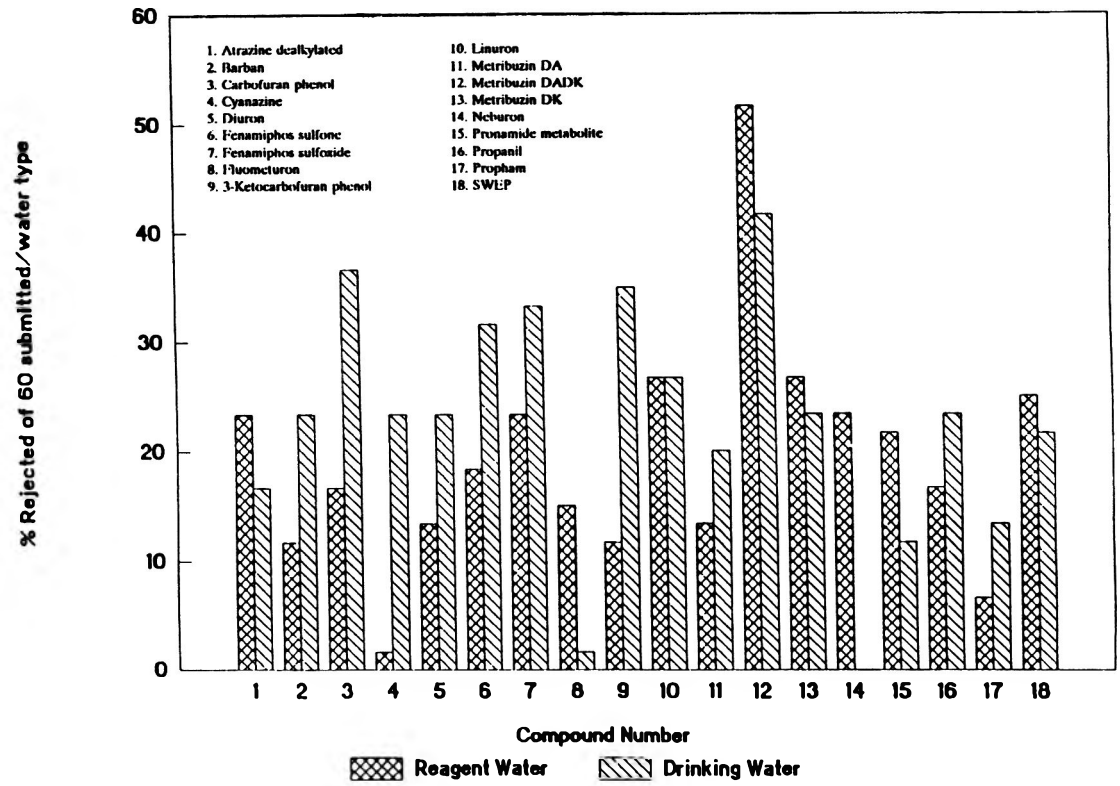

Figure 1. Percent rejected data by compound and water type.

Conclusions

Method 4, developed for the analysis of 18 pesticides in waters, was shown to be accurate and precise for most of the analytes in this collaborative study. Equations are presented for method recovery, overall precision, and single-analyst precision that can be used to estimate method performance at any concentration value within the study range. The recovery, overall precision, and single-analyst precision values were generally comparable in reagent water and finished drinking water, except for carbofuran phenol, which was found to have significantly higher matrix effects in finished drinking water than in reagent water.

The method recoveries for metribuzin DADK and metribuzin DK were very poor; however, the between-laboratory precision of these compounds was acceptable. These findings were consistent with previously published evaluations of the method (1).

Efforts should be made to optimize the resolution between closely eluting analytes to improve the quality of the resulting data. Adjustments to the mobile phase may be needed depending on the analytes present and the matrix type sampled.

The data were suitable for the development of performance-based QC limits. We recommend that users of this method routinely test a QC sample prepared in reagent water and compare the results with the performance-based acceptance limits derived in this study (Table **992.14C**).

Recommendation

On the basis of this interlaboratory method validation study, we recommend that the method be adopted first action.

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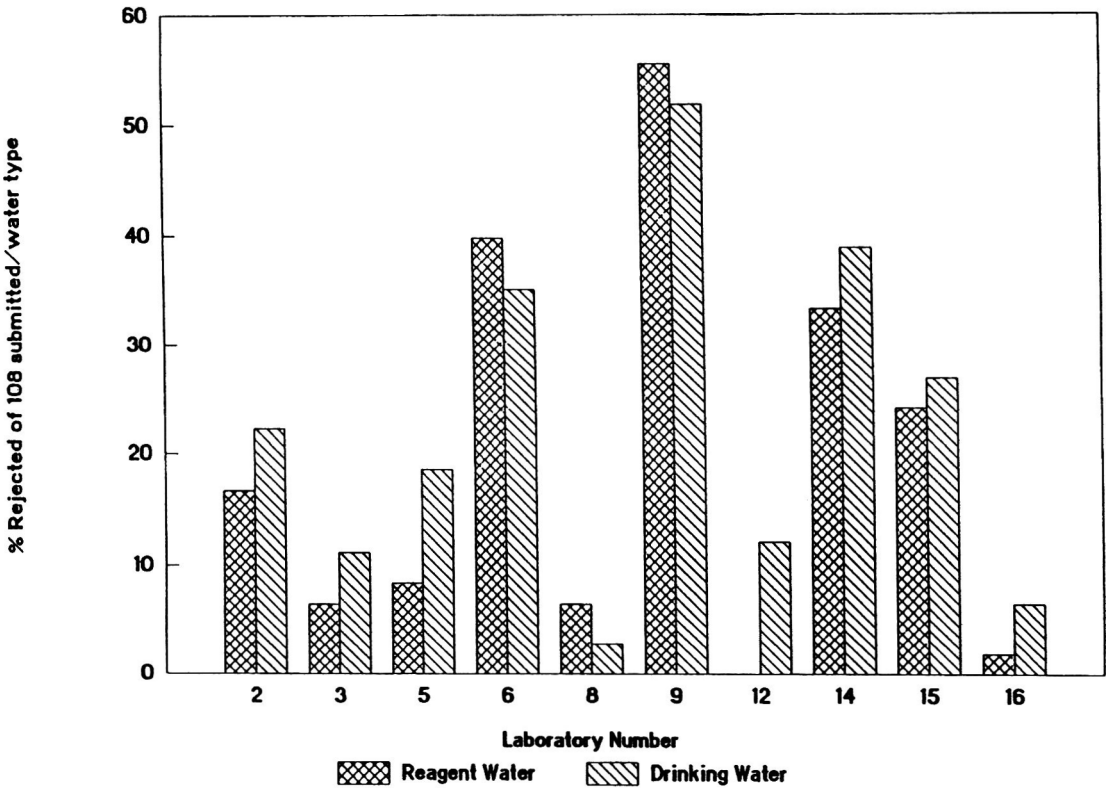

Figure 2. Percent rejected data by laboratory and water type.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Determination of Polynuclear Aromatic Hydrocarbons in Seafood by Liquid Chromatography with Fluorescence Detection

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Modification of a previously published method for determination of polynuclear aromatic hydrocarbons (PAHs) produces very clean seafood extracts in less than half the time. After alkaline digestion of the seafood, PAHs were partitioned into 1,1,2-trichlorotrifluoroethane. The resulting extract was cleaned up by solid-phase extraction on alumina, silica, and C_{18} adsorbents and then analyzed by gradient reversed-phase liquid chromatography with programmable fluorescence detection. Average recoveries of 12 PAHs [acenaphthene, anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)-fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene, benzo(ghi)perylene, and indeno(1,2,3-cd)pyrene] from 5 different matrixes (mussels, oysters, clams, crabmeat, and salmon) spiked at low parts-per-billion levels ranged from 76 to 94%. Estimated limits of quantitation ranged from 0.01 to 0.6 ppb PAHs in extracts that were free of matrix interferences. Results of analyses of a mussels standard reference material obtained from the National Institute of Standards and Technology were in good agreement with the certified values.

Polynuclear aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants that are of concern because of their mutagenicity and carcinogenicity (1, 2). Because PAHs are low-level impurities in crude oil, the Exxon Valdez oil spill in Alaska in the spring of 1989 prompted a renewed interest by the U.S. Food and Drug Administration in developing a rapid, reliable method for the determination of PAHs in seafood. Determining PAHs in environmental samples is a difficult analytical problem because of the large number of compounds and their low levels of occurrence.

Both gas chromatography (GC) and liquid chromatography (LC) are suitable techniques, but each has advantages and disadvantages. Capillary GC coupled with mass spectrometry (MS) (3–5) has the advantages of high selectivity, sensitivity, and resolving power and, therefore, can be used to determine a very large number of PAHs in a single analysis. Reversed-phase LC with fluorescence detection (6–10) is also very sensitive and selective, but it cannot match the high resolution of capillary GC. However, LC with fluorescence detection is generally more sensitive, particularly for PAHs of high molecular weight (11). Programmable fluorescence detection, in which the fluorescence excitation and emission wavelengths are changed during chromatographic separation, can be used to optimize quantitation of each PAH (12, 13).

Determination of PAHs in biological specimens requires isolation of PAHs from complex matrixes. Historically, cleanup procedures have been quite time-consuming, consisting of preparation of adsorbents, several hours of KOH digestion, many liquid-liquid extractions, and slow column cleanups (5–10, 14, 15). Recently Krahn et al. (4) used methylene chloride extraction, open-column chromatography on silica and alumina, and preparative scale, size exclusion chromatography to isolate PAHs. However, whether extraction completely removes PAHs from undigested biological tissues is uncertain (14). Although size exclusion cleanup is quite rapid, the column is very expensive. In addition, this method still requires slow open-column cleanup.

This paper reports a rapid method for routine determination of PAHs (naphthalene [Naph], acenaphthene [Ace], fluorene [Fl], phenanthrene [Phen], anthracene [Anth], fluoranthene [F], pyrene [Py], benz(a)anthracene [BaA], chrysene [Chr], benzo(b)fluoranthene [BbF], benzo(k)fluoranthene [BkF], benzo(a)pyrene [BaP], dibenz(a,h)anthracene [DBahA], benzo(ghi)perylene [BghiP], and indeno(1,2,3-cd)pyrene [IcdPy]) in seafood. It is a modification of the method of Joe et al. (7) in which liquid-liquid partition and open-column chromatography have been replaced by the more rapid solid-phase extraction (SPE). Seafood extracts are analyzed by reversed-phase LC with programmable fluorescence detection. Recoveries of 12 PAHs from mussels, oysters, clams, crabmeat, and salmon fortified at 3 different levels were determined. The method was also used to analyze a mussels standard reference material obtained from the National Institute of Standards and Technology (NIST).

METHOD

Apparatus

(a) *Liquid chromatograph.*—Model 8700XR solvent delivery system and Model 8880 autosampler (Spectra-Physics,

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Piscataway, NJ 08854), 4.6 × 250 mm Vydac 201TP54 column (The Separations Group, Hesperia, CA 92345), Model 470 scanning fluorescence detector (Millipore Corp., Waters Chromatography Div., Milford, MA 01757), Model 4400 integrator (Spectra-Physics), and WINner 386 data system (Spectra-Physics).

(b) *Digestion apparatus.*—500 mL round-bottom flask in 500 mL heating mantle (Cat. No. 721000-500, Kontes, Vineland, NJ 08360) used with variable transformer (Cat. No. 730900) set at 50% output. Insert reflux condenser (Cat. No. 457000-0175) in round-bottom flask.

(c) *Special glassware.*—Pear-shaped flasks, 300 mL (Kontes Cat. No. 608700-0524) and 50 mL (Cat. No. 294260-0050); Buchner funnel with coarse-porosity fritted disk (Cat. No. 955000-0623); and 10 mL graduated concentrator tube (Cat. No. 570050-1025).

(d) *Flash evaporator.*—Used with 40°C water bath (Buchler Instruments, Inc., Fort Lee, NJ 07024).

(e) *Extraction manifold.*—Baker-10 SPE system (J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

(f) *Mixer.*— Vortex-Genie (Scientific Industries, Inc., Bohemia, NY 11716).

Reagents and Experimental Materials

(a) *Solvents.*—High purity hexane, methylene chloride, acetonitrile, and water (Baxter Healthcare Corp., Burdick & Jackson Div., Muskegon, MI 49442); 1,1,2-trichlorotrifluoroethane (TCTFE) (E.I. du Pont de Nemours & Co., Wilmington, DE 19898); and anhydrous ethanol (The Warner-Graham Co., Cockeysville, MD 21030). TCTFE and ethanol were distilled before use.

(b) *Ethanolic KOH solution.*—Dissolve 40 g KOH in 1 L ethanol.

(c) *Sodium sulfate.*—Anhydrous, granular (EM Science, Gibbstown, NJ 08027).

(d) *SPE columns.*—Alumina, 6 mL high capacity (HC); silica, 6 mL; and C_{18}, 6 mL HC (Baker).

(e) *Filter.*—Nylon-66 syringe filter unit, 0.45 μm, 13 mm diameter (Rainin Instrument Co., Inc., Woburn, MA 01801).

(f) *PAH standard solutions.*—Solution 1.—Standard Reference Material 1647 (U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD 20899). Contains 16 PAHs in acetonitrile (μg/mL): Naph (19.9), acenaphthylene (15.9), Ace (19.6), Fl (4.76), Phen (3.37), Anth (0.771), F (7.68), Py (8.25), BaA (3.82), Chr (3.62), BbF (4.08), BkF (4.61), BaP (4.82), DBahA (3.72), BghiP (3.75), and IcdPy (4.32). Solution 2.—Dilute Solution 1 with acetonitrile (1 + 24). Dilute Solution 2 to obtain working standards.

Extraction and Cleanup

(**Note**: Rinse all glassware with appropriate solvent before use.) Accurately weigh 10 g homogenized seafood into 500 mL round-bottom flask. Add 100 mL ethanolic KOH solution and few boiling chips and digest 2 h. Cool to room temperature. Pour solution into 500 mL separatory funnel. Rinse round-bottom flask sequentially with 90 mL water, 50 mL ethanol, and 40 mL TCTFE. Add rinsings to separatory funnel. Extract by rigorous shaking for 2 min. Drain TCTFE (lower) layer through Buchner funnel containing 50 g Na_2SO_4 (prerinsed with 40 mL TCTFE) into 300 mL pear-shaped flask. Repeat extraction with 2 additional 40 mL portions of TCTFE; pass each extract through Na_2SO_4 into pear-shaped flask. Rinse Na_2SO_4 with 40 mL TCTFE and add rinsings to flask. Force residual solvent through Na_2SO_4 into flask by pressing heal of hand over funnel. Concentrate to ca 10 mL on flash evaporator with 40°C water bath. Add 10 mL hexane and concentrate to ca 1 mL.

Rinse alumina SPE column with 20 mL methylene chloride followed by 2 mL hexane. Do not let column become dry. Rinse silica column in similar manner, but do not use vacuum; let solvent pass through column under gravity. Insert silica column into top of extraction manifold. Position alumina column on top of silica column. Transfer concentrated extract from pear-shaped flask to alumina column with disposable Pasteur pipet. Rinse flask with two 1 mL portions of hexane and add rinsings to alumina column. Use pressure from N_2 cylinder to force extract and rinsings through alumina column; use vacuum to draw extract through silica column. Elute PAHs from columns with three 5 mL portions of methylene chloride–hexane (1 + 3), using N_2 pressure for alumina column and vacuum for silica column. Do not let columns become dry until after last portion of eluting solvent has been added. Collect eluate in 50 mL pear-shaped flask. Add 4 mL CH_3CN and concentrate contents of flask on flash evaporator to ca 1 mL.

Condition C_{18} SPE column with 20 mL CH_3CN. Transfer extract to column with disposable pipet. Rinse flask with two 1 mL portions of CH_3CN, transfer rinsings to column, and apply vacuum. Elute column under vacuum with three 5 mL portions of CH_3CN and collect eluate in 50 mL pear-shaped flask. Do not let column become dry until last portion of eluting solvent has been added. Concentrate eluate to ca 1 mL on flash evaporator. Transfer concentrated extract to 10 mL concentrator tube. Rinse flask with 2–3 mL CH_3CN and add rinsings to concentrator tube. Concentrate extract under N_2 to <1 mL in 40°C water bath, and adjust volume to 1.0 mL with CH_3CN. Mix thoroughly on vortex mixer and pass extract through Nylon-66 filter. If LC analysis cannot be conducted immediately, store extract in refrigerator.

Chromatographic Determination

Inject 20 μL aliquots of extract and standard of suitable concentration into liquid chromatograph, using 1.0 mL/min flow rate and the following solvent program: 3 min hold at 50% CH_3CN–50% water, linear gradient to 100% CH_3CN in 15 min, and 8 min hold at 100% CH_3CN. (The fluorescence detector program is given in Table 1.) Calculate concentration of each PAH from following equation (assume final extract volume of 1.0 mL):

$$\text{PAH (ppb)} = (H_{ext}/H_{std})(C_{std}/W)$$

where H_{ext} and H_{std} = chromatographic peak heights obtained for PAH in extract and standard, respectively; C_{std} = concentra-

Table 1. Detector program used to determine PAHs

No.[a]	PAH	Wavelength, nm		Gain
		Excitation	Emission	
1	Naph	270	350	10
2	Ace	270	350	10
3	Fl	270	350	10
4	Phen	270	350	10
5	Anth	250	420	10
6	F	250	420	10
7	Py	250	420	10
8	BaA	270	390	10
9	Chr	270	390	10
10	BbF	290	410	10
11	BkF	290	410	10
12	BaP	290	410	10
13	DBahA	290	410	10
14	BghiP	300	465	100
15	IcdPy	300	465	100

[a] Number identifies peak in Figures 1–3.

tion (ng/mL) of PAH in standard; and W = weight (g) of test portion.

Results and Discussion

The first 2 steps of the cleanup, i.e., alkaline digestion and partition with TCTFE, are similar to those in the original method (7). However, one minor change was found to be quite important. The original method called for placing KOH pellets and ethanol in the digestion flask. However, the KOH does not totally dissolve until the ethanol begins to heat up in the heating mantle.

During the analysis of a reagent blank, the solid KOH browned or charred in the initial stages of heating; the final extract was yellow. The chromatogram of the blank showed many peaks, some of which interfered with PAH determination. Figure 1 shows chromatograms of 2 reagent blanks, one in which KOH was predissolved in ethanol and one in which solid KOH was added. Predissolving the KOH results in a chromatogram with a much lower background and is, therefore, specified in the Method section.

Digestion of the seafood is the most time-consuming step of the method; the subsequent cleanup steps may be completed in 1–2 h. Silica–alumina cleanup, which is performed on disposable SPE columns, is much faster than the gravity column cleanup used in the original method; in addition, the lengthy preparation of adsorbents called for in the original method is avoided. Furthermore, solvent consumption is much lower for SPE columns. The complicated series of dimethyl sulfoxide–cyclohexane partitions, back extractions, and water washes in the original method is replaced by SPE on a disposable C_{18} column, which also saves both time and solvents.

Figure 2 shows the chromatogram, obtained under the described conditions, of a 1 + 249 (v/v) dilution of NIST Standard Reference Material 1647 that contains 16 PAHs. (One PAH in the mixture, acenaphthylene, does not fluoresce.) The first few peaks in the chromatogram are slightly asymmetric because 100% CH_3CN is a stronger eluting solvent than the initial mobile phase (50% CH_3CN–50% water). When seafood extracts

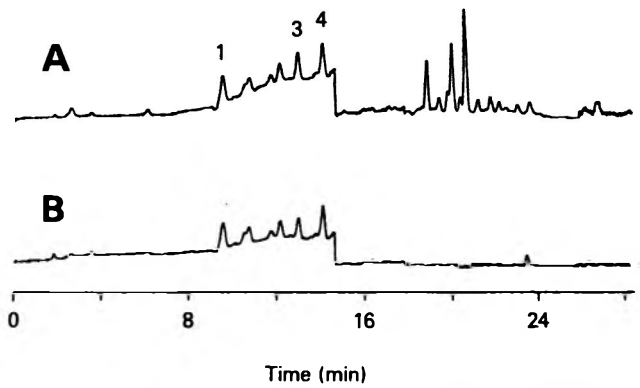

Figure 1. Chromatograms of reagent blanks. (A) KOH not dissolved in ethanol before starting digestion and (B) KOH predissolved in ethanol. Conditions are given in Method section. Numbered peaks are identified in Table 1.

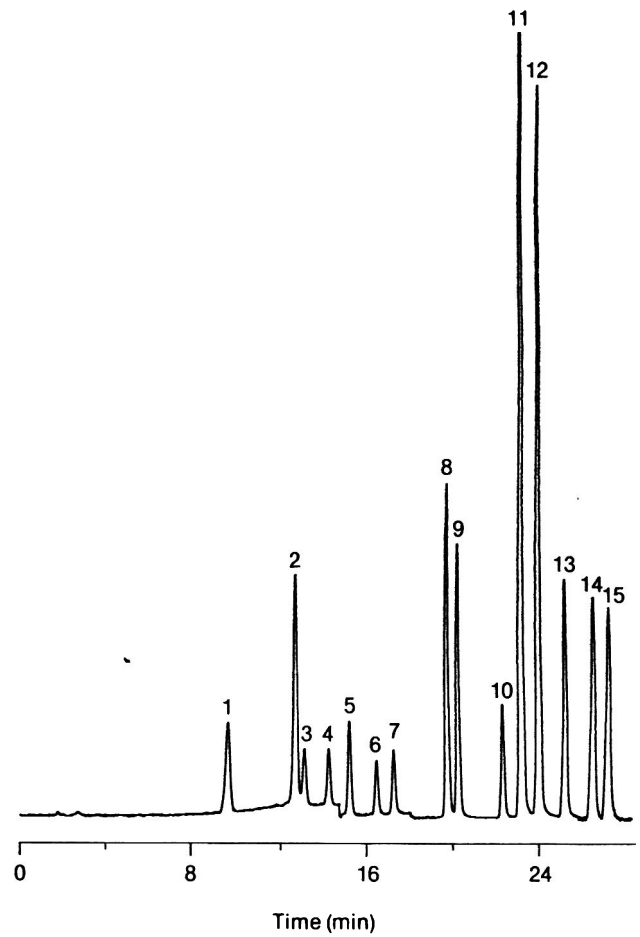

Figure 2. Chromatogram of PAH standard. Numbered peaks are identified in Table 1. Concentrations range from 3 ng/mL for anthracene to 80 ng/mL for naphthalene. Conditions are given in Method section.

Table 2. Average recoveries and CVs of PAHs from fortified seafood[a]

PAH	Level, ppb	Av. rec., % (CV, %)				
		Mussels	Clams	Oysters	Crabmeat	Salmon
Ace	7.84	74.5 (2.6)	76.9 (4.3)	77.0 (6.7)	76.9 (3.6)	66.8 (5.6)
Anth	0.308	81.4 (5.1)	83.8 (4.0)	83.5 (3.5)	82.5 (3.5)	78.1 (3.8)
F	3.07	82.4 (6.9)	84.6 (4.6)	81.5 (6.4)	88.1 (7.7)	88.9 (4.7)
Py	3.30	88.0 (5.3)	95.5 (9.6)	94.3 (6.9)	89.7 (7.0)	88.4 (4.4)
BaA	1.53	89.1 (3.9)	88.3 (2.6)	93.9 (5.1)	91.3 (2.0)	89.0 (2.5)
Chr	1.45	85.3 (4.6)	85.7 (2.1)	90.7 (3.6)	91.0 (3.3)	85.7 (2.8)
BbF	1.63	90.8 (2.8)	91.1 (2.1)	93.2 (2.4)	93.3 (2.8)	83.5 (3.6)
BkF	1.84	91.0 (2.2)	92.6 (1.9)	93.6 (1.6)	91.8 (2.2)	83.6 (3.8)
BaP	1.93	90.8 (2.3)	90.8 (1.7)	92.5 (2.3)	91.4 (3.0)	80.5 (3.2)
DBahA	1.49	89.2 (2.7)	91.8 (3.1)	95.3 (2.3)	85.6 (8.0)	78.8 (7.8)
BghiP	1.50	94.1 (9.1)	93.7 (8.8)	94.3 (3.4)	92.8 (14.1)	—
IcdPy	1.73	91.7 (7.4)	93.7 (2.9)	96.8 (2.0)	89.1 (5.5)	—

[a] n = 12.

were diluted with water, some of the high molecular weight PAHs were lost during the filtration step, probably as a result of the very low water solubilities of these compounds. Thus, for accurate determination of the high molecular weight PAHs, less than ideal chromatography of the low molecular weight PAHs was accepted.

Recoveries of PAHs from clams, mussels, oysters, crabmeat, and salmon were determined. Seafood was spiked with the appropriate amount of PAH Standard Solution 2 just after weighing. Six replicate test portions of each matrix were analyzed for recovery and variation at the levels listed in Table 2. In addition, 3 replicates at half and 3 at twice these levels were analyzed, for a total of 12 test portions for each matrix. Because recovery was independent of spiking level, all 12 determinations were averaged, and matrix response was subtracted before recovery was calculated. Chromatograms for fortified and unfortified clams are shown in Figure 3. Chromatographic peaks representing low levels of presumed PAH were observed in chromatograms for unfortified clams; similar peaks were observed in the chromatograms for unfortified mussels and oysters.

Recoveries did not vary much among matrixes, with the exception of salmon. Percentage recoveries ranging from the mid-80s to the low 90s were obtained for most PAHs, with somewhat lower recoveries obtained for the more volatile Ace and Anth. These results are comparable to those obtained by Joe et al. (7) with the original method. For salmon, recoveries were as much as 11% lower than those for the other matrixes. During preparation of salmon, an oily film was observed on the glassware. This phenomenon was not observed during the preparation of shellfish, and might indicate incomplete saponification. Some PAH material may have been trapped in this oily layer and not recovered by the method. Interferences at the retention times of BghiP and IcdPy in the salmon extracts made quantitation of these chromatographic peaks impossible.

The results of the recovery study indicate that the method is generally successful for quantitating low levels of individual PAHs that range from 0.15 ppb Anth to 4 ppb Ace. The calculated limit of quantitation (LOQ), defined as 10 standard deviations above the average blank signal (16), is lower than the tested level for all PAHs. Several reagent blanks were run, and the noise levels at appropriate points in the chromatograms

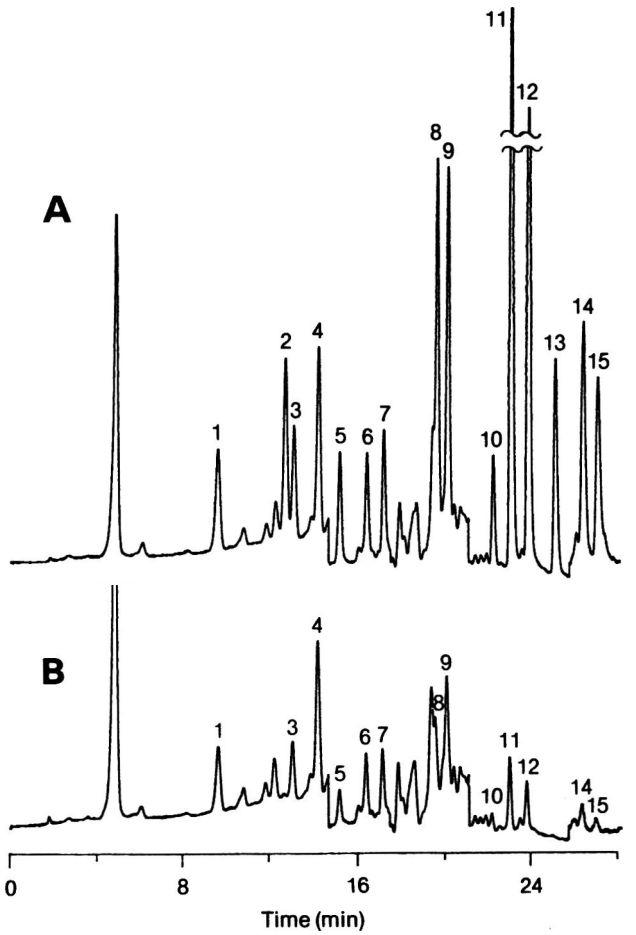

Figure 3. Chromatograms from analysis of (A) fortified and (B) unfortified clams. Numbered peaks are identified in Table 1; fortification levels are given in Table 2. Conditions are given in Method section.

Table 3. Limits of quantitation (LOQ) for PAHs[a]

PAH	LOQ, ng/mL
Naph	5.96
Ace	2.25
Fl	2.09
Phen	1.70
Anth	0.20
F	3.64
Py	3.59
BaA	0.30
Chr	0.35
BbF	0.89
BkF	0.12
BaP	0.17
DBahA	0.36
BghiP	1.20
IcdPy	1.36

[a] Obtained by using wavelengths in Table 1.

were measured. The LOQs were calculated and then converted to concentration units (Table 3). Each LOQ in parts per billion may be calculated by multiplying the corresponding value in Table 3 by the final extract volume (mL) and dividing by the weight of the test portion (g). Thus, for a 10 g test portion and a final volume of 1.0 mL, the LOQ for BaP is 0.017 ppb. These LOQs should be considered as the lowest concentrations that can be reliably quantitated in the absence of matrix interferences. If an interference from the matrix is unresolved or a reagent blank gives a response, the actual LOQ will be higher than the value given in Table 3.

Table 2 does not include data for Naph, Fl, and Phen because the responses for these 3 low molecular weight PAHs in the reagent blanks were too high (Figure 1). Ethanol contained low levels of these 3 PAHs that were not eliminated by distillation. The SPE columns also produced traces of these PAHs, even though the columns were rinsed with large volumes of solvent before use. Work on this problem is continuing.

In addition to analyzing fortified seafood, we used the method to analyze a mussels standard reference material (NIST SRM 1974). Table 4 compares the results of duplicate analyses with the NIST-certified values. Results were not corrected for recovery; thus, the somewhat low results for Phen and Py are reasonable. Otherwise, the agreement between the values determined by the method and those determined by the certified values are good. Determination of perylene required a separate analysis, because it coelutes with BbF under the conditions given in the Method section. However, when a fluorescence excitation wavelength of 400 nm and an emission wavelength of 500 nm are used, perylene fluoresces but BbF does not.

Several features of the fluorescence detector used in this study greatly facilitate PAH determination. First, the detector is very sensitive. Second, both excitation and emission wavelengths can be set to increase specificity and selectivity. PAHs that coelute, e.g., perylene and BbF, may often be quantitated by suitable adjustment of these wavelengths. Third, because the detector is programmable, many PAHs may be determined

Table 4. PAHs in mussel standard reference material 1974

	Concentration, ppb		
PAH	NIST certified	Found	
Phen	5.6 ± 1.4	3.99	4.07
Anth	0.75 ± 0.21	0.72	0.72
F	33.6 ± 5.8	32.5	32.8
Py	34.1 ± 3.7	29.5	29.1
Perylene	1.05 ± 0.29	0.95	1.00
BbF	6.5 ± 1.2	5.93	5.91
BaP	2.29 ± 0.47	2.20	2.25
BghiP	2.47 ± 0.28	2.32	2.36
IcdPy	1.80 ± 0.33	1.61	1.65

in a single injection. It should be emphasized that, although such a detector is desirable, its use is not essential to the method. Less sophisticated fluorescence detectors can be used; however, the size of the test portion may need to be increased to have adequate detector response for quantitation. Mussel test portions 2–3 times as large as those called for in the method (10 g) have been successfully analyzed. Overloading of the SPE columns was not a problem, and recoveries were comparable with those obtained with 10 g.

In summary, a rapid, reliable method for the determination of PAHs in seafood has been developed. It produces fairly clean extracts, even for complex matrixes such as bivalves. Recoveries from test samples fortified at low parts-per-billion levels are about 90% for most PAHs. Work on improving the method (e.g., eliminating background) and extending the method to other food matrixes is continuing.

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PESTICIDE FORMULATIONS

Determination and Confirmation of *cis*-9-Tricosene in Technical Materials by Capillary Gas Chromatography and Gas Chromatography/Mass Spectrometry

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A capillary gas chromatographic (GC) method was developed for the quantitation of *cis*-9-tricosene in technical material. This method is capable of resolving *cis*-9-tricosene from the *trans*-9-tricosene isomer and other impurities in technical 9-tricosene. Samples are dissolved in methylene chloride and analyzed by splitless GC using docosane as an internal standard. The integrity and purity of the peak of interest were confirmed by GC/mass spectrometry. The overall recovery of this method was 101.3 ± 0.82%. The correlation coefficient of the standard calibration curve was 0.9999. The system and method precision for *cis*-9-tricosene were 0.18 and 0.20%, respectively. The reproducibility of the method by different analysts was within 0.5%.

The compound *cis*-9-tricosene (Figure 1), a sex attractant pheromone of the common housefly, *Musca domestica* L., was first isolated from the cuticle and feces of the female housefly (1–5). The synthetic *cis*-9-tricosene prepared later (6–9) is now widely used in formulations with insecticide to attract and control the fly population. Previously reported analytical methods for *cis*-9-tricosene have mainly been concerned with determination of double bond location, structure identification (10–12), derivatives of *cis*-9-tricosene (13–16), and bioassay (17). In the present paper, we describe a validated method using splitless capillary gas chromatography (GC) with *n*-docosane as an internal standard for the quantitation of *cis*-9-tricosene in synthetic material without interferences from *trans*-9-tricosene and other impurities. GC/mass spectrometry (GC/MS) and GC/infrared (GC/IR) detection were also used to confirm and identify the chromatographic peaks of *cis*- and *trans*-9-tricosene.

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Experimental

Apparatus

(a) *Gas chromatograph.*—Model 5890A gas chromatograph equipped with flame ionization detector, splitless injector, Model 3392A integrator, Model 3350 lab data system, and Model 7673A autosampler (Hewlett-Packard Company, Avondale, PA 19311). Operating conditions: injector temperature, 250°C; detector temperature, 300°C; injection mode, splitless injection with splitless silica liner; linear velocity (helium), 37 cm/s at 150°C; hydrogen, 40 mL/min; air, 400 mL/min; make up gas (helium), 30 mL/min; injection volume, 1 μL; oven temperatures: initial, 160°C; rate, 1°C/min; final, 170°C; stop time, 60 min.

(b) *Chromatographic column.*—30 m × 0.32 mm capillary DB-1 column with 0.25 μm film thickness (J&W Scientific, Folsom, CA). Three columns from different lots were tested for reproducibility (Lot No. 0231974, 0267126, and 2031976).

(c) *Gas chromatograph/mass spectrometer.*—Model 5985B (Hewlett-Packard) with GC/LC/MS data system operated in positive electron ionization mode under the following conditions: electron multiplier, 2.0 kV; ion source temperature, 200°C; emission current, 300 μA; GC operating conditions: same as (a) and (b).

(d) *Glassware.*—20 and 50 mL repipettors fitted to 4 gallon amber bottle; 100 mL amber bottles with polyethylene-lined caps; 25 and 50 mL Class A volumetric flasks; 1 mL glass GC vials; Class A volumetric pipets.

(e) *Gas supply.*—Hydrogen, air, and helium with suitable molecular sieve filters, 2-stage regulator, and flow controllers.

Reagents

(a) *cis-9-Tricosene standard.*—Supplied by Aldrich Chemical Co., Milwaukee, WI, Lot 05120TV, at 97.3% purity.

(b) *Docosane internal standard.*—Supplied by Aldrich Chemical Co., Lot 01017EW, at 99% purity.

(c) *Dichloromethane.*—Optima grade (Fisher Scientific, Pittsburgh, PA 15219).

(d) *trans-9-Tricosene.*—The *trans*-9-tricosene used for this study was prepared from isomerization of *cis*-9-tricosene standard by modified procedure of Henrick et al. (18) as follows:

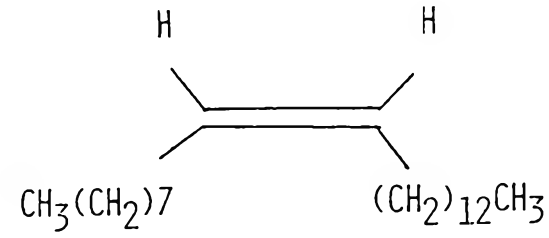

Figure 1. Molecular structure of *cis*-9-tricosene.

Weigh 0.10 g *cis*-9-tricosene standard in 50 mL flask, add 0.4 mL thiophenol, and reflux mixture under nitrogen at 160°C for 1 h. Add another 0.4 mL thiophenol and continue heating for 0.5 h. Dilute reaction mixture with 50 mL hexane and determine by GC as mixture of *cis*- and *trans*-isomers in 17:83 ratio. Confirm peak purity of *trans*-9-tricosene by GC/MS (Figure 2).

To further identify the structure of the *trans* isomer, GC/IR was used for analysis. The existence of characteristic IR absorption at 967 cm^{-1} was used to confirm the *trans*-isomer peak. Structure information obtained from this experiment was consistent with structure information for isolated *trans*-9-tricosene from Sandoz Crop Protection, CA, and previously reported values (1).

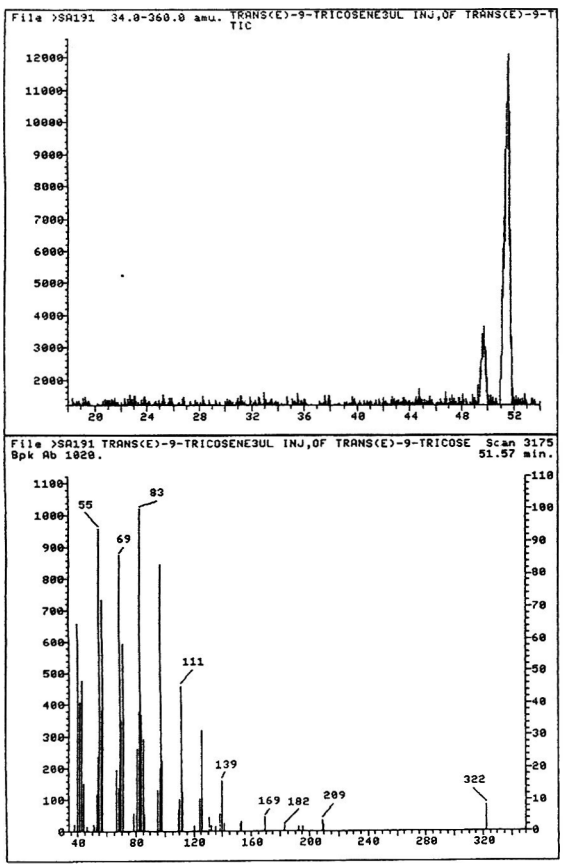

Figure 2. Representative gas chromatogram of synthetic *trans*-9-tricosene detected by mass spectrometer (top). The mass spectrum of *trans*-9-tricosene at retention time of 51.57 min (bottom).

(e) *Technical cis-9-tricosene.*—Lot A and Lot B from Sandoz Crop Protection, Palo Alto, CA; Lot C from Orsynex, Inc.

Preparation of Standards and Samples

(a) *n-Docosane internal solution.*—Accurately weigh 0.1500 g 99% *n*-docosane in 100 mL volumetric flask and dilute to volume with methylene chloride (1.50 mg/mL).

(b) *cis-9-Tricosene stock solution.*—Accurately weigh 0.1600 g 97.3% reference *cis*-9-tricosene into 100.0 mL volumetric flask, and dilute to volume with methylene chloride (1.56 mg/mL).

(c) *cis-9-Tricosene standard–internal standard solution for sample assay.*—Pipet 20.0 mL methylene chloride with repipettor in 100 mL amber jar, and add 1.0 mL *cis*-9-tricosene stock solution and 1.0 mL internal standard solution. Shake well.

(d) *Sample preparation.*—Quantitatively weigh 0.2600 g technical *cis*-9-tricosene in 100 mL amber jar, add 100.0 mL methylene chloride, and shake well to dissolve. Pipet 1.0 mL sample solution into 100 mL amber jar, and add 1.0 mL internal standard solution and 20.0 mL methylene chloride. Use this sample solution for analysis.

Determination

Inject 1 µL standard solution until standard ratios (*SR*) reproduce to within ±1%. Precede sample analysis by 3 injections of standard. Report assay results as follows:

$$\%\ cis\text{-9-tricosene} = (VR/SR) \times (Ws/W) \times \%\ \text{purity of standard} \times 100$$

where *SR* is ratio of response of standard to that of internal standard, and *VR* (variable ratio) is ratio of response of *cis*-9-tricosene to that of internal standard. All data collected were based on area responses. *Ws* and *W* are weights (g) of reference standard and sample, respectively.

Results and Discussion

Resolution and System Suitability

Resolution.—The developed chromatographic system was capable of resolving from the analyte peak all other components that will give a detector signal; this includes internal standard, *trans*-9-tricosene, and other impurities. The resolution value, *R*, between *cis*-9-tricosene and *trans*-9-tricosene was 1.57; *R* between *cis*-9-tricosene and *n*-docosane (internal standard) was 11 when calculated as follows:

$$R = [2(t_2 - t_1)]/(W_1 + W_2)$$

where t_1 and t_2 are retention times of 2 measured peaks, and W_1 and W_2 are peak widths of 2 peaks, respectively.

Repeatability.—The chromatographic system demonstrated high repeatability. The relative standard deviation

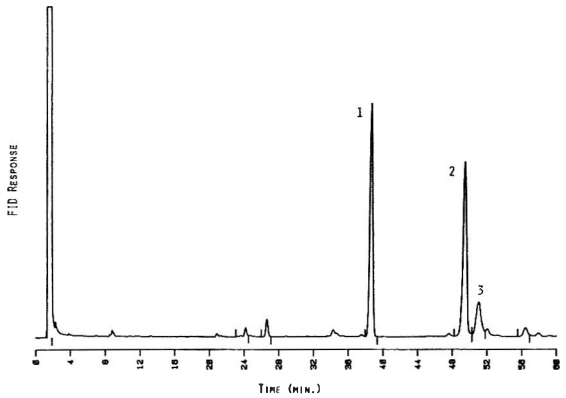

Figure 3. Representative gas chromatogram of technical *cis*-9-tricosene detected by flame ionization detector. Peak No. 1, internal standard, *n*-docosane; peak No. 2, *cis*-9-tricosene; peak No. 3, *trans*-9-tricosene.

(RSD) of peak response ratios from the analysis of 6 replicates of standard solutions was 0.18%.

Tailing factor.—The tailing factors of *cis*-9-tricosene and *n*-docosane (internal standard) were 0.968 and 1.12, respectively, when calculated as follows:

$$T = 0.05W/2f$$

where T is tailing factor, $0.05W$ is the peak width at 5% peak height, and f is the distance from the leading edge to the peak maximum.

Linearity

Linearity for cis-9-tricosene.—A series of *cis*-9-tricosene standard solutions (0–114.8 μg/mL) containing the same concentration of internal standard (60 μg/mL) was prepared to establish the system linearity. Each concentration level was injected 6 times. The SR from the injections plotted against the theoretical concentration gave a regression coefficient of 0.99995.

Linearity for internal standard.—To establish the linearity for *n*-docosane (internal standard), 6 levels of concentration ranging from 0 to 117 μg/mL were prepared, and each level was injected 3 times. A plot of area counts vs the theoretical concentration of the internal standard gave a regression coefficient of 0.99982.

Specificity

To test the system suitability of this method for future stability studies, 3 lots of technical *cis*-9-tricosene were stressed at 75°C for 2 weeks, and samples were analyzed at 1 and 2 weeks. The chromatograms obtained by using flame ionization detection showed no additional peaks that interfere with the peak of *cis*-9-tricosene (Figure 3). The integrity and purity of the analyte peak was also verified by GC/MS at the same chromatographic conditions. The peak purity was checked by taking mass spectra from the leading, tailing, and central areas of the analyte peak. The molecular ion at $m/z = 322$ and major ion fragmentations at $m/z = 111, 97, 55$, etc., for reference and technical *cis*-9-tricosene were consistent (Figure 4). The peak that elutes just after the *cis*-9-tricosene in technical material was also identified as *trans*-9-tricosene by comparison with retention time, IR spectra, and its major ion fragmentations (1). The peak purity of stressed samples was also confirmed by GC/MS and showed no coelution with the analyte.

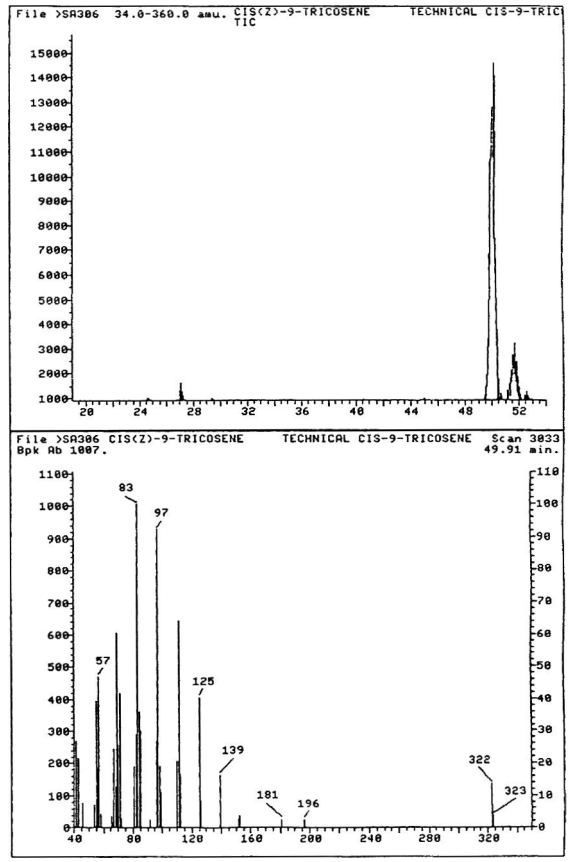

Figure 4. Representative gas chromatogram of technical *cis*-9-tricosene detected by mass spectrometer (top). The mass spectrum of *cis*-9-tricosene at retention time of 49.81 min (bottom).

Precision

The system precision for *cis*-9-tricosene was established by preparing 6 replicates of standard–internal standard solution at the concentration equivalent to 100% of technical and analyzing 3 times for each sample. The average RSD is 0.18%. The method precision was determined by analyzing duplicate samples from 3 different lots of technical *cis*-9-tricosene. Each sample solution also containing internal standard was analyzed 3 times. The average RSD for the sample assays was 0.20%.

Accuracy

The standard addition method was used for the determination of method accuracy for *cis*-9-tricosene. Six replicates of technical *cis*-9-tricosene were quantitatively weighed from

Table 1. Percent recovery of *cis*-9-tricosene from fortified technical materials

Lot	Rec., %	SD
Lot A	101.8	0.45
Lot B	101.5	0.74
Lot C	100.6	0.75
Mean	101.3	0.82

each of 3 different lots in 100 mL amber jars. A 0.030 g portion of reference *cis*-9-tricosene standard and 100.0 mL methylene chloride were added to each. The samples were then assayed. The percent recoveries for Lots A, B, and C are shown in Table 1. The theoretical values (%) in Table 1 were calculated according to the following equation:

$$\text{Theoretical } \% = [Ws\ (\text{g}) \times I\ (\%) + A\ (\text{g}) \times \%\ \text{purity of standard}] \times 100/wt\ (\text{g})$$

where *Ws* (g) is the amount of sample weighed, *A* (g) is the amount of reference standard added, *wt* (g) is the total weight, and *I* (%) is the initial value from the analysis of 6 replicates of homogeneous technical *cis*-9-tricosene (19).

Sensitivity

The sensitivity of a method is defined as the slope of the calibration. The limit of detection (LOD) of a method is calculated within a section of the plot close to the origin by using the determined (y) intercept and slope.

System sensitivity.—Standard analyte solutions with internal standard using at least 7 concentrations ranging from 10 to 200% of label declaration were prepared. Each sample was analyzed 3 times. The limit of detection was calculated as follows:

$$\text{System detection limit, XLod} = (\text{System YLod} - a)/b = 0.778\ \mu\text{g/mL}$$

where System YLod = $a + 3Sxy$; a = intercept = 0.0046309; b = slope = 0.01563; and Sxy = standard deviation about regression line = 0.0040526. This calculated system detection limit was consistent with the detection limit that was determined by signal-to-noise ratio of ca 3:1.

Method sensitivity.—The limit of detection of the method was determined by preparing the sample solution as for system sensitivity except that technical *cis*-9-tricosene was used instead of reference *cis*-9-tricosene. The calculated limit of quantitation was 1.91 µg/mL, which was equivalent to 0.017 g *cis*-9-tricosene/g of technical material. This calculated limit of quantitation was also consistent with the quantitation limit that was determined by signal-to-noise ratio of ca 10:1.

Ruggedness

Stability of analyte solution.—To evaluate the stability of standard analyte solutions and sample analyte solutions, the RSDs from assays of fresh and aged samples (70 h later) were compared. The response ratios of standard and technical samples were as follows: fresh standard–internal solution, 1.025 ± 0.001; aged standard–internal standard solution, 1.021 ± 0.004; fresh technical–internal standard solution, 1.001 ± 0.002; and aged technical–internal standard solution, 0.9973 ± 0.003.

Column-to-column variability.—The column-to-column variability was determined by analyzing 3 different lots of technical *cis*-9-tricosene and reference standard using 3 different lots of capillary GC columns. The average retention time of *cis*-9-tricosene from 3 different column lots was 49.65 ± 0.17 min (RSD = 0.34%). The average retention time of *n*-docosane (internal standard) from 3 different column lots was 38.84 ± 0.12 min (RSD = 0.31%).

Reproducibility

To verify the reproducibility of the method, the assays for system and method precision were repeated by different analysts. The results of the first analyst were compared with those of the second analyst according to the following equation (20) for the coefficient of variation (CV):

$$\%\text{CV} = 2^{(1 - 0.5 \log C)}$$

System precision.—Six replicates of reference *cis*-9-tricosene were analyzed 3 times. The system RSD for the second analyst was 0.42%.

Method precision.—Duplicate samples from 3 different lots of technical *cis*-9-tricosene were assayed in triplicate. The method RSD for the second analyst was 0.46%. The theoretical %CV for 60% technical *cis*-9-tricosene was 2.15% on the basis of the following equation:

$$\%\text{CV} = 2^{(1 - 0.5 \log C)}$$

All RSD values from different analysts were less than 1/2 %CV (1.43%), a result that demonstrates good reproducibility (20).

Conclusion

Using splitless capillary GC with a methylsilicone DB-1 column under gradient oven temperature, we were able to produce a highly reproducible and precise method for the determination of *cis*-9-tricosene in technical material. The purity of the chromatographic peaks of *cis*- and *trans*-9-tricosene was positively confirmed by GC/MS. This validated method, which provides high chromatographic resolution, is suitable for product chemistry and stability studies of technical *cis*-9-tricosene.

Acknowledgments

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SEAFOOD TOXINS

Indirect Competitive Enzyme Immunoassay for Tetrodotoxin Using a Biotin–Avidin System

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An indirect competitive enzyme immunoassay was developed to determine tetrodotoxin (TTX). Antiserum against TTX was demonstrated in rabbits immunized with TTX–keyhole limpet hemocyanin conjugate. In this assay, TTX–bovine serum albumin was coated on the microtiter plate, which was incubated with standard TTX and biotinylated immunoglobulin G from antiserum. The amount of antibody bound on the surface of wells was determined by the reaction of avidin–peroxidase conjugate with its substrate. The minimal detection level of TTX ranged from 5 to 25 pg/assay. The standard curve was linear between 0.25 and 50 ng/assay. A good correlation ($r = 0.974$) for TTX was also obtained between this indirect competitive enzyme immunoassay and the mouse bioassay.

Tetrodotoxin (TTX) is one of the most potent nonprotein poisons. The toxin is predominantly isolated from the ovary and liver of several species of puffer fish (1, 2) and is also detected in some other animal species (3–6). More recently, TTX was also isolated from some shellfish in Japan (7–9). The ingestion of toxic puffer fish has resulted in paralytic poisoning and occasional death in humans. The toxicology and pharmacology of this toxin have been reviewed by Kao (10).

TTX can be measured by various methods, such as thin-layer chromatography, cellulose acetate membrane electrophoresis, nuclear magnetic resonance spectrometry (7), capillary isotachophoresis (11), and liquid chromatography (12). Because of its simplicity, the mouse bioassay has been used as an official method for the quantitation of TTX (13). One mouse unit (MU) is described as that amount of TTX that kills a male ddY mouse weighing 19–20 g within 30 min (equivalent to 0.2 μg). However, the bioassay shows low precision and also requires a continuous supply of mice.

This study describes an indirect competitive enzyme immunoassay (EIA) for the detection of TTX. Keyhole limpet hemocyanin (KLH) was used as a carrier protein and was conjugated to TTX in the presence of formaldehyde. High-titer antiserum against TTX was elicited in rabbits immunized with this immunogen. Subsequently, this antiserum could be used to prepare a highly sensitive EIA system. Furthermore, picogram concentrations of TTX per assay could be detected, and a good correlation for TTX could be obtained between the mouse bioassay and the EIA technique.

Received July 9, 1991. Accepted February 7, 1992.

Experimental

Materials

TTX (crystalline 3X) was obtained from Sankyo Co., Ltd (Tokyo, Japan). Tween 20, *o*-phenylenediamine (OPD), 30% hydrogen peroxide, and ammonium sulfate were obtained from Wako Pure Chemical Co. (Tokyo, Japan). Goat anti-rabbit immunoglobulin G (IgG)–peroxidase conjugate was purchased from Cappel Laboratories (West Chester, PA). *N*-Hydroxysuccinimidobiotin was obtained from Pierce Chemical Co. (Rockford, IL). Horseradish peroxidase avidin-D was obtained from Vector Laboratories, Inc. (Burlingame, CA). Bovine serum albumin (BSA) was purchased from Miles Inc. (Kankakee, IL). KLH was obtained from Calbiochem Co. (La Jolla, CA). Complete Freund's adjuvant and gelatin were purchased from Difco Laboratories (Detroit, MI). Microtiter plates were obtained from Greiner Labortechnik (Nurtingen, Germany).

Female albino rabbits and male ddY mice were purchased from Shizuoka Laboratory Animal Research (Hamamatsu, Japan). Specimens of the puffer fish, *Takifugu niphobles*, which were caught off the Seto Inland Sea in May 1991, were supplied from a local seafood store.

Preparation of TTX–KLH Complex (Antigen)

The antigen (TTX–HCHO–KLH) was prepared according to the method of Chu and Fan (14). In a typical experiment, 6.0 mg KLH in 2 mL 0.05M phosphate buffered saline (PBS), pH 8.0, reacted with 1.0 mg TTX in the presence of 0.04 mL 37% formaldehyde. The reaction was carried out at room temperature for 72 h and then at 4°C for 12 h. The reaction mixture was dialyzed against 4 L 0.02M PBS at 4°C for 72 h with 4 changes of the PBS to remove residual free TTX. The molar ratios of TTX to KLH were estimated to be ca 1100:1 from the decrement of free amino groups before and after the reaction (15). The same method was used to prepare TTX–HCHO–

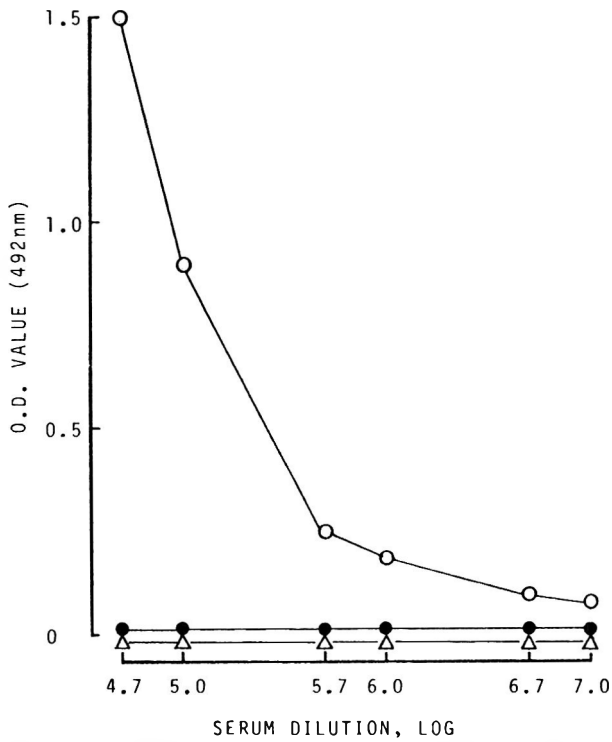

Figure 1. Titration of antibody titer by ELISA. Titer of antisera from rabbits immunized with TTX–HCHO–KLH (○) and KLH (●) using TTX–HCHO–BSA coating plate and the reactivity of anti-TTX–HCHO–KLH against BSA coating plate (△).

BSA. The molar ratio of TTX to BSA in this preparation was ca 18:1.

Immunization

Antiserum to TTX was elicited in 2 albino female rabbits immunized with an emulsion prepared from 1.0 mg antigen (TTX–HCHO–KLH) in 1.0 mL PBS with an equal volume of complete Freund's adjuvant by multiple subcutaneous injections. Six weeks after the initial immunization, 10 booster injections were given at biweekly intervals in the same manner. Ten days after the last injection, the rabbit was bled and the serum was separated. The same schedule was followed for producing the antiserum to KLH used as a control serum throughout this study. IgG fraction from the antiserum to TTX–KLH was prepared by 33% ammonium sulfate precipitation; after removal of the salt, the precipitate was passed through a DEAE–cellulose column. Part of this IgG fraction was biotinylated by the method of Bayer et al. (16).

Enzyme Immunoassay

Serum titration.—The enzyme-linked immunosorbent assay (ELISA) was used for the serum titration, as follows: 100 μL TTX–HCHO–BSA or BSA at 1.0 μg/mL in 0.5M carbonate buffer, pH 9.6, was added to each well of a microtiter plate that had been sensitized by overnight incubation at 4°C. The antigen was removed, and unbound sites in the wells were blocked with 200 μL 0.1% gelatin in 0.02M PBS, pH 7.2. The plate was incubated for 1 h at room temperature, then the blocking buffer was removed, and the plate was washed 3 times with PBS containing 0.05% Tween 20. Next, 100 μL of various dilutions of antiserum was added to each well and incubated 2 h at 37°C. The antiserum was removed, and the plate was washed again. Then, 100 μL goat anti-rabbit IgG peroxidase conjugate (1:10000 dilution in PBS) was added to each well and incubated 1 h at 37°C. The conjugate was removed, and the plate was washed 4 times. To each well was added 100 μL substrate solution (OPD plus hydrogen peroxide in phosphate–citrate buffer, pH 5.0) that was freshly prepared before use, and the plate was incubated for 10 min at room temperature. The enzyme reaction was stopped with 50 μL 2.5M H_2SO_4, and the plate was read in a spectrophotometer at 492 nm.

Quantitation of TTX.—Indirect competitive EIA was used to quantitate TTX. Antigen coating and blocking of wells were carried out as before. After washing, 50 μL TTX standards or samples were added to each well, followed by 50 μL biotinylated IgG from antiserum to TTX at 0.25 μg/mL in PBS, and the contents of the well were immediately mixed with microtiter mixer. The plate was incubated 2 h at 37°C, removed, and washed. Next, 100 μL avidin–peroxidase conjugate that was optimally diluted in PBS was added, and the plate was incubated 1 h at 37°C. The amount of bound antibody was determined as in the ELISA procedure.

Preparation of Samples

Samples from puffer fish were prepared by acetic acid–methanol extraction (13). To 5 g homogenized puffer fish muscle, 25 mL acetic acid–methanol was added. The homogenate was heated 10 min at 70°C and then filtered. The operation was repeated 3 times. The filtrates were combined, concentrated under reduced pressure, and defatted with ethyl ether twice. The aqueous layer was diluted with water to a total volume of 10 mL. This solution was used for mouse bioassay, which was performed by the method of Kawabata (13). For use in the indirect competitive EIA, the solution was diluted 1:10 in 0.02M PBS, pH 7.2. To compare the mouse bioassay with the EIA procedure, the results of this study were expressed as TTX content (i.e., 1 MU = 0.22 μg TTX).

Results and Discussion

The TTX–KLH conjugate synthesized as an immunogen gave a good immunological response in rabbits. Figure 1 shows a typical titration curve obtained by an ELISA procedure for the antibody titer for a rabbit immunized with TTX–HCHO–KLH. When TTX–HCHO–BSA was coated on the microtiter wells, highly diluted antiserum had a high optical density (OD), whereas antiserum to KLH had no reaction. The low dilution (1:10–1:10000) of antiserum to TTX had an OD value of >3.0 in this ELISA (data not shown). When BSA was coated as an antigen, the antisera to both TTX–HCHO–KLH and KLH were negative. These results confirm that the antiserum to TTX–HCHO–KLH is TTX-specific and has no reaction with BSA.

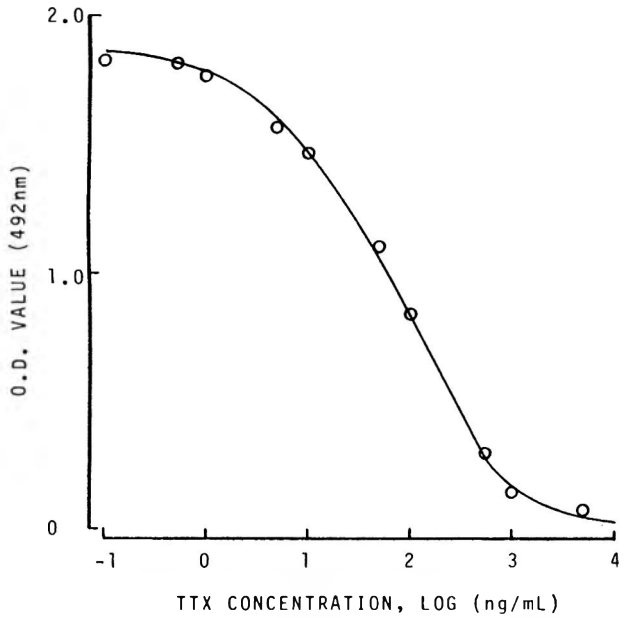

Figure 2. Typical standard curve for TTX in indirect competitive EIA. The competition of TTX and biotinylated IgG was carried out, followed by avidin–peroxidase conjugate. In this system, biotinylated IgG and avidin–peroxidase were used at 0.25 μg/mL and 1:10000 dilution, respectively.

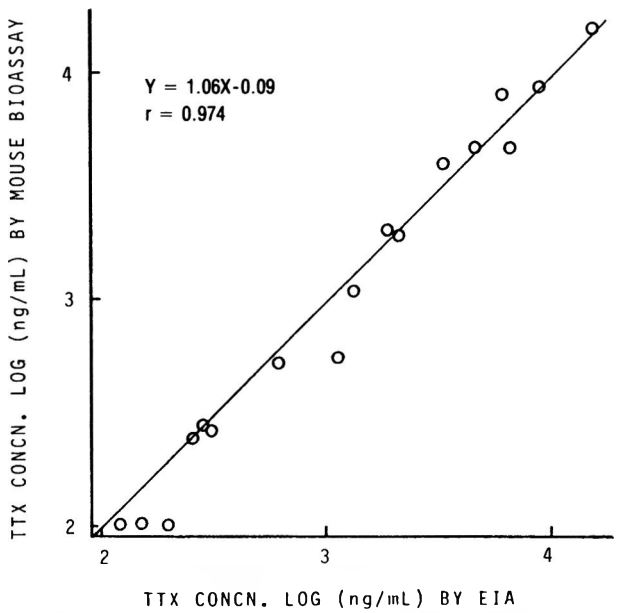

Figure 3. Correlation of results for TTX obtained by mouse bioassay and by indirect competitive EIA (*n* = 17).

In this study, our efforts concentrated on producing antibody with a high affinity to TTX. However, Ragelis (17) claimed that it was difficult to produce an antibody with a good titer for TTX in rabbit challenged with TTX–BSA conjugate through 1-carbon spacer binding. Watabe (18) also reported that the antibody titer obtained from mice immunized with TTX–HCHO–KLH was low, whereas we have obtained a highly elevated antibody to TTX (OD >2.0 at 1:10000 dilution) even in mice challenged with the same antigen (data not shown). The results presented here indicate that TTX–HCHO–KLH is a good immunogen for TTX in both rabbits and mice.

Table 1. Estimates of within-assay and between-assay variabilities of standard curves for indirect competitive EIA for TTX

TTX concn, ng/mL	Mean ± SD, ng/mL	CV, %
	Within-assay	
500	500.0 ± 46.0	9.2
100	108.5 ± 9.0	8.3
50	46.5 ± 7.3	15.7
10	10.8 ± 1.4	13.1
	Between-assay	
500	502.5 ± 76.7	15.3
100	105.0 ± 14.7	14.0
50	54.0 ± 11.5	21.3
10	10.7 ± 1.6	15.0

A typical standard curve for the indirect competitive EIA for TTX using a biotin–avidin coupled-enzyme system is presented in Figure 2. Under the experimental conditions, the OD values of the blanks (a mixture of 50 μL each of PBS and biotinylated IgG) ranged from 1.75 to 1.95. The apparent lower limits for TTX detection by this assay ranged from 5 to 25 pg/assay (100–500 pg/mL). However, the curve was linear for TTX doses between 0.25 and 50 ng/assay (5–1000 μg/mL). When the sample contains a high level of TTX, it must be diluted more than 1:10.

The EIA technique has been developed for some seafood toxins such as saxitoxin (14) and ciguatoxin (19). An ELISA technique for TTX using monoclonal antibody was reported by Watabe et al. (20), who stated that the reactivity of the antibody was rather low and the detection limit was 0.2 μg/assay. However, because the reactivity of antiserum produced by small molecules is generally weak compared with that of protein antigen, we biotinylated IgG to enhance the EIA technique. The sensitivity of the biotin–avidin system is greater than or equal to those of a radioimmunoassay and a fluorescence immunoassay technique (21, 22). Although a 1:5000 dilution of the antiserum (equivalent to about 2 μg IgG/mL) was used in the preliminary study with a regular indirect competitive EIA system, IgG at 0.25 μg/mL could be applied to this EIA technique after biotinylation of the IgG. When the antiserum was used at 0.25 μg IgG/mL without biotinylation, TTX could not be measured in the regular method because of low OD (data not shown).

The within-assay and between-assay variations from the standard curve are shown in Table 1. The within-assay and between-assay coefficients of variation were less than 15.7 and 21.3%, respectively. Thus, a sensitive and reproducible assay has been produced with a wide enough range for practical purposes.

Figure 3 shows the results obtained from each puffer fish sample by mouse bioassay plotted against the results obtained by indirect competitive EIA. The correlation coefficient was 0.974 for the 17 samples. In the calculation for the negative results (first 3 points) by mouse bioassay, 0.1 μg/mL each was tentatively substituted for the 3 TTX contents obtained by the EIA system. However, the correlation of results obtained by the 2 methods, based on different principles (in vivo and in vitro), seems to be excellent.

It is well known that the TTX itself has several derivatives (23). A recently published observation also showed that puffer fish contain not only TTX but also saxitoxin and other toxic substances (24). These facts require further study of the cross-reactivity of antibody used here. We are currently working on the production of monoclonal antibody in the hope of decreasing cross-reactivity and increasing specificity.

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VITAMINS AND OTHER NUTRIENTS

Determination of Total Vitamin C in Various Food Matrixes by Liquid Chromatography and Fluorescence Detection

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A liquid chromatographic method was developed for quantitation of total vitamin C content in various food matrixes. The method includes extraction with 3% *meta*-phosphoric acid–acetic acid and oxidation of ascorbic acid to dehydroascorbic acid with Norit, followed by reaction with *o*-phenylenediamine to form a fluorescent derivative. The fluorescent derivative is separated on a μBondapak C_{18} (10 μm) column with methanol–water (55 + 45) and detected fluorometrically. This method is demonstrated to be suitable for several sample matrixes, including complex matrixes of canned corn, potatoes, green beans, potato chips, and cereals. Other products analyzed include infant formula, medical foods, fruit juice, and multivitamin tablets. Recoveries are in the range of 90–108%. Comparison of results with those by the AOAC fluorometric method shows excellent agreement.

Vitamin C has long been recognized as an important nutrient in several food products. The reduced form of the vitamin is referred to as ascorbic acid (AA), and the oxidized form is referred to as dehydroascorbic acid (DHAA). In humans, both forms are biologically active. The total vitamin C activity is the sum of both forms.

Analytical methods for the determination of vitamin C include the 2,6-dichloroindophenol titrimetric method (1), the 2,4-dinitrophenylhydrazine colorimetric method (2), the microfluorometric method (3, 4), and several liquid chromatographic (LC) methods. The titrimetric and colorimetric methods lack specificity and are subject to matrix interferences that can seriously hamper the accuracy of measurement. The AOAC microfluorometric method (4) is specific for total vitamin C and is applicable to products considered to be good sources of vitamin C. For foods with naturally occurring low levels of vitamin C, a larger sample size is required, which increases the likelihood of matrix interference.

Difficulties are also encountered when analyzing food products with high starch contents, high fat contents, and/or intense coloration. These interferences result in either cloudy extracts and/or high blanks. As reported by Augustin et al. (5), unrealistically high values were obtained when the microfluorometric method was applied to potatoes and potato products. The extraction procedure had to be modified to remove the interfering substances before the determination could be completed.

Although improved LC methods are replacing existing methods, review of all the published articles and reports is beyond the scope of this paper. Instead, selected relevant references have been cited. Some of these methods quantitate only ascorbic acid. Sood et al. (6) used a reversed-phase ion-pairing LC system with UV detection at 254 nm to determine ascorbic acid in food and multivitamin preparations. Augustin et al. (5) modified the method of Sood et al. by using 95% ethanol in the sample preparation to precipitate the starch in potatoes and potato products before LC separation. Ashoor et al. (7) used an ion-exchange LC system with UV detection at 254 nm to determine ascorbic acid in a variety of food products.

These methods are limited because vitamin C may occur naturally in many foods as AA and DHAA. Unless DHAA is converted to AA, misleading low results may be obtained when only the reduced form of vitamin C is measured. Vanderslice et al. (8) examined a large number of food products and found that ≥90% of the products contained DHAA at different levels. In some products such as coleslaw, fresh carrots, and potatoes with skin, DHAA levels may represent about 40% or more of total vitamin C content.

Other LC methods have been developed for the determination of both vitamers. Wimalasiri and Wills (9) and Finley and Duang (10) used UV detection at 254 and 210 nm to measure AA and DHAA, respectively. When the concentration of DHAA is low, UV detection at 210 nm is not sensitive enough to accurately measure DHAA because of interference from the solvents at low wavelengths. Derivatization techniques using precolumn or postcolumn systems enhance the sensitivity of DHAA measurements by UV or fluorescence detection. Keating and Haddad (11) produced a DHAA–OPDA derivative for increased sensitivity in analyzing orange juice. Behrens and Madere (12) used DL-homocysteine to convert DHAA to AA with subsequent electrochemical detection. Kim (13) first reduced DHAA to AA with dithiothreitol and then measured total vitamin C in several food products by ion exclusion chromatography with electrochemical detection. Vanderslice and Higgs (14) used anion-exchange chromatography with postcolumn oxidation of AA to DHAA followed by derivatiza-

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tion with OPDA and fluorescence detection. The method required a specialized postcolumn reaction system but was applicable to several food products.

Our objective was to improve the AOAC microfluorometric method. We have developed a relatively simple, versatile, and sensitive method for quantitating total vitamin C in various products. This method can be used for high and low potency samples and has overcome the effects of matrix interference, eliminating the need for blank corrections and additional cleanup steps.

METHOD

Reagents

(a) *Extracting solution.*—3% *meta*-Phosphoric acid in 8% acetic acid solution. Prepare by dissolving 15 g HPO_3 pellets in 40 mL glacial acetic acid and 200 mL water; dilute to 500 mL with water and filter rapidly through fluted paper into glass-stoppered bottle. Stored in refrigerator, solution is satisfactory 7–10 days (J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

(b) *Ascorbic acid standard solution, 1 mg/mL.*—Accurately weigh 50 mg L-ascorbic acid standard (J.T. Baker Chemical Co.) into 50 mL volumetric flask. Dilute to volume immediately before use with extracting solution.

(c) *Working standard, 100 µg/mL.—Dilute 10.0 mL L-ascorbic acid standard solution to 100.0 mL with extracting solution.*

(d) *Sodium acetate solution.*—Dissolve 500 g NaOAc·$3H_2O$ in water and dilute to 1 L. Store in plastic bottle.

(e) *Acid-washed Norit.*—Add 1 L HCl (1 + 9) to 200 g Norit Neutral (carbon decolorizing, C-170, Fisher Scientific Co.). Heat to boiling point and filter under vacuum. Transfer cake to 2 L beaker. Add 1 L H_2O, stir, and filter. Repeat washing with H_2O and filtering until neutral to pH. Dry overnight at 110–120°C and store in plastic bottle.

(f) *Methanol.*—Distilled-in-glass (Baxter Healthcare Corp., Burdick and Jackson Division, Muskegon, MI 49442). High purity solvent suitable for LC analysis.

(g) *Mobile phase.*—Methanol–water (55 + 45). Mix 550 mL methanol with 450 mL distilled water. Filter through 0.5 µm Millipore filter, sonicate 5 min to degas, and allow solution to come to room temperature before use.

(h) *o-Phenylenediamine (OPDA) solution, 2.5 mg/mL.*—Dissolve 250 mg *o*-phenylenediamine dihydrochloride (Eastman Kodak Co., Rochester, NY 14650), and dilute to 100 mL with mobile phase immediately before use.

Chromatographic Apparatus

(a) *LC system.*—Model 6000A pump, Model U6K injector, and µBondapak C_{18} column, 30 cm × 3.9 mm (Waters Associates, Milford, MA 01757).

(b) *Detector.*—Model 650-10LC fluorescence detector with variable wavelength and adjustable slits; set sensitivity range at 1.0, slit at 7 mm, excitation wavelength at 350 nm, and emission wavelength at 430 nm (Perkin-Elmer, Norwalk, CT). HP 3390A integrator (Hewlett-Packard, Palo Alto, CA). Allow system to equilibrate for ca 30 min by pumping mobile phase at flow rate of 1.0 mL/min.

Samples

Most of the study samples were purchased from local supermarkets. Usually, one consumer-size portion was used for analysis. If more than one consumer-size portion was needed to complete study, only samples from same lot were used.

Sample Preparation

Prepare sample composites under subdued or yellow light, and protect from oxidation by processing and mixing under nitrogen. Prepare composites of liquid and powdered materials by combining and thoroughly mixing contents of sample subunits. Prepare composites of solid or semisolid materials, such as frozen vegetables, by homogenization in food processor or heavy duty blender under nitrogen atmosphere. Immediately after composite preparation, weigh analytical sample portion (to contain 1.5–7.5 mg vitamin C), and mix with extracting solution to avoid loss of vitamin C. Designate preparation as sample assay solution; preparation is stable and suitable for overnight storage under refrigeration. Warm to room temperature before use.

(a) *Liquid material.*—Accurately weigh appropriate analytical sample portion into 100 mL volumetric flask, and dilute to volume with extracting solution.

(b) *Dry material (powders).*—Accurately weigh analytical sample portion into 100.0 mL volumetric flask, add ca 50 mL extracting solution, gently swirl until sample dissolves (if sample does not readily dissolve, sonicate for ca 5 min), and dilute to volume with extracting solution.

(c) *Solid or semisolid material.*—Accurately weigh analytical sample portion into glass-stoppered graduated cylinder. Add ca 40 mL extracting solution, shake vigorously, and centrifuge for 5 min at 6000 rpm. Quantitatively transfer supernatant into 100 mL volumetric flask. Extract residue again with another 40 mL portion of extracting solution. Centrifuge, transfer supernatant to original 100 mL volumetric flask, and dilute to volume with extracting solution.

Sample Determination

Perform the following treatment steps for each sample assay solution consecutively to complete determination on same day. Protect from light by using low actinic glassware or wrapping containers in aluminum foil.

(a) *Conversion to DHAA.*—Transfer entire contents of 100 mL volumetric flask of each sample assay solution to 250 mL glass-stoppered Erlenmeyer flask containing 2 g acid-washed Norit. Shake vigorously for 30 s, filter (under vacuum or gravity) through Whatman No. 40 (ashless) filter paper, and discard first few milliliters. Designate Norit-treated filtrate as DHAA preparation.

(b) *Clarification of DHAA preparation.*—Transfer 20 mL aliquot of Norit-treated filtrate (or any appropriate volume to contain ca 300–700 µg vitamin C) to 100 mL volumetric flask containing 5 mL sodium acetate solution and 55 mL methanol, dilute to volume with water, and shake well to mix. Filter

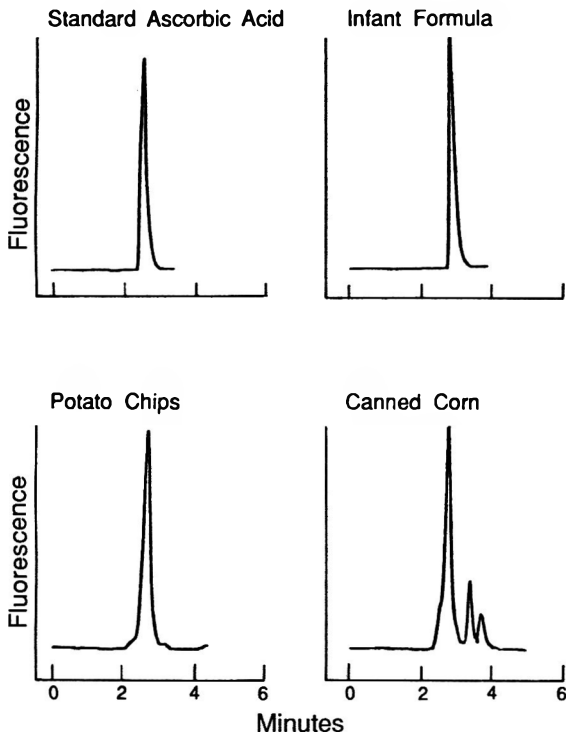

Figure 1. Chromatograms of DHAA–OPDA derivative of vitamin C from standard solution, infant formula, potato chips, and canned corn samples using μBondapak C_{18} column and methanol–water (55 + 45) mobile phase at a flow rate of 1 mL/min. Peaks were monitored at 350 nm excitation and 430 nm emission wavelengths.

through Whatman No. 40 filter paper or centrifuge for 5 min at 6000 rpm.

(c) *Formation of DHAA–OPDA derivative.*—Transfer aliquot of clarified DHAA preparation containing ca 150 μg vitamin C to 100 mL volumetric flask. Add 10 mL OPDA solution, dilute to volume with mobile phase, shake well, and let stand in dark for 60 min at room temperature. Developed fluorescent DHAA–OPDA derivative is stable for ca 6 h. Inject 100 μL aliquots in duplicate for quantitation of total vitamin C.

Standard Curve

Treat 100 mL working standard vitamin C preparation (100 μg/mL) as described under (a) and (b) for *Sample Determination.* Dispense 0, 2.0, 5.0, 10.0, 15.0, and 25.0 mL of the clarified standard DHAA solution into respectively designated 100 mL volumetric flasks. Prepare DHAA–OPDA derivative as described under (c) for *Sample Determination.* Final concentrations of standard preparations are 0, 0.4, 1.0, 2.0, 3.0, and 5.0 μg vitamin C/mL, respectively.

Calculation

Calculate concentration of total vitamin C present in sample by linear regression equation from standard curve and report as mg/100 g sample.

Comparison with AOAC Method

This LC method was compared with the AOAC microfluorometric method (4). However, products that contained naturally very low levels of vitamin C, high starch and/or fat content, or intense coloration required modification of the AOAC extraction before the formation of DHAA–OPDA derivative. Such modification was necessary to eliminate sample matrix interferences.

Modified AOAC Extraction Procedure

After obtaining Norit-treated filtrate (AOAC, Step F, for vitamin C by microfluorometric method) (1), dilute 25 mL aliquot of each sample and standard solution filtrate with equal volume of 95% ethanol, place in ice bath for 0.5 h, and centrifuge. Transfer 10 mL aliquots of clear filtrates to corresponding volumetric flasks and proceed per AOAC instructions starting with ... "contg 5 mL H_3BO_3–NaOAc ...".

Recovery Studies

Vitamin C was added to each sample in amounts equivalent to the declared level before processing. The percent recovery was calculated by dividing the amount of added vitamin C found by the actual amount added and multiplying by 100.

Results and Discussion

Fluorometric detection of total vitamin C content requires conversion of ascorbic acid to dehydroascorbic acid before formation of the fluorescent DHAA–OPDA derivative. Figure 1 shows the baseline-resolved vitamin C peaks of the standard, infant formula, potato chips, and canned corn samples. Vitamin C eluted after about 2.9 min.

To establish that the fluorescence response is due only to the DHAA–OPDA derivative and not to other interfering substances, a peak purity evaluation was performed as described by Haroon et al. (15). The evaluation involved comparison of the fluorescence ratio of the standard and the sample at 2 or more specific excitation wavelengths while the emission wavelength was kept constant. In this study, fluorescence responses at excitation wavelengths of 330, 350, and 370 nm were used to calculate peak height ratios of 350:330 and 350:370 at a constant emission wavelength of 430 nm. Table 1 shows the ratios obtained with the standard, canned corn, and canned potatoes. Close agreement of the ratios for the standard and samples indicated that sample vitamin C peaks were homogeneous. Method repeatability was evaluated by analyzing spiked infant formula sample representing high levels of vitamin C and spiked canned corn sample representing low vitamin C levels. Each sample was analyzed 5 times; the mean ± standard deviation (SD) and the percent coefficient of variation (% CV) were 188.2 ± 3.15 mg/100 g (% CV = 1.67) and 14.85 ± 0.16 mg/100 g (% CV = 1.08) for infant formula and canned corn, respectively. These results demonstrate good reproducibility at both levels.

Fluorescence response was directly proportional to the concentration of the standard over the range 0.138–3.0 μg/mL.

Table 1. Evaluation of vitamin C peak purity by fluorescence response ratio[a]

Product	Peak response ratio 350/330 nm	Peak response ratio 350/370 nm
Ascorbic acid standard	1.55 ± 0.04	1.32 ± 0.03
Canned corn	1.51 ± 0.02	1.30 ± 0.03
Canned potatoes	1.42 ± 0.02	1.27 ± 0.02

[a] Each value is the mean ± SD and is based on triplicate assays/sample and 6 replicates/standard.

The concentration range used for most samples was 0.8–2.5 μg/mL. The limit of detection for total vitamin C was about 10 ng/100 μL injection.

Results of vitamin C analysis by the LC and the AOAC microfluorometric methods are summarized in Table 2. The 10 samples used to evaluate and compare the 2 methods represent a wide variety of sample matrixes. The samples also represent food products containing unfortified (naturally occurring low levels) and fortified levels of vitamin C. Each value (expressed as mg/100 g) is the mean ± SD of duplicate determinations. Results obtained by the LC method were comparable with those obtained by the AOAC method, including samples that required modification of the AOAC extraction, with a correlation coefficient of 0.9995. Samples containing naturally occurring low levels of vitamin C, high starch contents, high fat contents, and/or intense color were difficult to analyze and gave unreliable results by the AOAC method because of sample matrix interferences. However, reliable results were obtained after treatment of Norit filtrates with equal amounts of 95% ethanol and filtration before development of DHAA–OPDA derivatives by the AOAC method.

Problem samples such as canned corn, canned potatoes, potato chips, and canned green beans were successfully analyzed by the LC method without additional time-consuming cleanup and the need for blank determinations as required by the AOAC method. Results for analysis of additional samples including orange juice, tomato juice, and frozen broccoli showed very good agreement between the 2 methods.

Table 2. Total vitamin C content of various products by the LC and AOAC microfluorometric methods[a]

Product	LC, mg/100 g	AOAC, mg/100 g
Infant formula (milk powder)	101 ± 0.0	104 ± 0.50
Medical food (liquid)	33.7 ± 0.1	30.5 ± 0.3
Potato chips	59.2 ± 0.6	62.2 ± 0.60[b]
Cereal (fortified)	211 ± 6.0	221 ± 1.0
Corn, canned	5.9 ± 0.02	5.6 ± 0.01[b]
Green beans, canned	2.9 ± 0.02	3.0 ± 0.02[b]
Potatoes, canned	0.6 ± 0.005	0.6 ± 0.002[b]
Cranberry juice	32.1 ± 0.1	32.1 ± 0.1
Cranapple juice	45.1 ± 0.05	41.4 ± 0.05
Multivitamin tablet	16480 ± 637	16072 ± 382

[a] LC values are the mean ± SD of duplicate injections from duplicate extractions. AOAC values are the mean ± SD of duplicate extractions.
[b] Modified AOAC extraction by use of 95% ethanol.

Table 3. Recovery of vitamin C by the LC and AOAC microfluorometric methods[a]

Product	Rec., % LC	Rec., % AOAC
Infant formula (milk powder)	95.4 ± 0.05	93.8 ± 1.35
Medical food (liquid)	89.9 ± 0.1	106 ± 0.5
Potato chips	104 ± 5.2	106 ± 2.5[b]
Cereal (fortified)	91.7 ± 1.1	103 ± 2.0
Corn, canned	91.1 ± 0.4	96.8 ± 0.4[b]
Green beans, canned	92.2 ± 4.45	97.2 ± 0.45[b]
Potatoes, canned	108 ± 1.5	90.4 ± 0.45[b]
Cranberry juice	94.8 ± 1.95	99.4 ± 1.65
Cranapple juice	100 ± 0.5	105 ± 1.0
Multivitamin tablet	89.9 ± 0.6	97.6 ± 1.2

[a] LC values are the mean ± SD of duplicate injections from duplicate extractions. AOAC values are the mean ± SD of duplicate extractions.
[b] Modified AOAC extraction by use of 95% ethanol.

Recovery values were determined by adding amounts of vitamin C equivalent to the declared level in the sample. Table 3 shows the percent recovery for 10 samples assayed by the LC and AOAC methods. The mean recoveries were 95.7 and 99.5% for the LC and AOAC methods, respectively. However, the good agreement between the results of the 2 methods was made possible by modifying the AOAC method for the samples that showed sample matrix interference. Further, the method does not differentiate between L-ascorbic acid and isoascorbic acid. In the event that isoascorbic acid is added as an antioxidant and appears on the ingredient listing, another method that quantitatively separates ascorbic acid from isoascorbic acid should be used, such as that described by Vanderslice and Higgs (14).

Conclusion

The LC method described here is shown to be accurate, precise, and specific for the quantitation of total vitamin C in various products. Quantitation is based on the measurement of a sharp single DHAA–OPDA peak that is shown to be free of all interfering substances. Complex samples in which matrix interference is apparent or samples with very low levels of vitamin C that require modification of the AOAC method before analysis are successfully analyzed by the described LC method. Meanwhile, the method requires no additional cleanup and eliminates the need for blank determination as required by the AOAC method. Furthermore, this study has shown that laboratories with limited accessibility to LC systems can use the modified AOAC extraction for vitamin C determination in problem samples such as canned corn, canned potatoes, potato chips, and green beans.

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VITAMINS AND OTHER NUTRIENTS

Liquid Chromatographic Determination of Folic Acid in Infant Formula and Adult Medical Nutritionals

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A liquid chromatographic method for the determination of pteroylglutamic acid (folic acid) in infant formula and liquid medical nutritional diets is described. Extraction of folic acid from the sample matrix is facilitated by a partial enzymatic digestion of the sample proteins. An aliquot of the sample preparation is injected onto a strong anion exchange column (SAX) for preliminary separation, using a pH 5.3 mobile phase of 0.02M sodium acetate and 0.02M sodium sulfate. Before the elution of the folic acid from the SAX column, the eluant is directed onto an octyl bonded-phase column (C_8) by using automated switching valves. After the folic acid has been transferred to the C_8 column, the SAX column is removed from the flow path. The folic acid is eluted from the C_8 column by using an acetonitrile gradient. Detection is by absorption at 345 nm. The mean coefficient of variation is 3.6%, and the range of the recoveries of added folic acid is 95.5–100.2%. The method detection limit and method quantitation limit for infant formula are 10 and 28 μg/kg, respectively. This method is used routinely for the determination of folic acid in infant formula and liquid medical nutritional diets.

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Infant formulas, liquid medical nutritional diets, and other products designed to provide total sustenance contain protein sources that contribute indigenous forms of the family of vitamers collectively referred to as folates. Because of uncertainty concerning the levels of the various folates in the ingredients and the known instability of many forms of folate under the conditions of thermal processing, such products are commonly fortified with a single, stable form of the vitamin to levels that comply with nutritional and regulatory requirements. Pteroylglutamic acid, or folic acid (1), is the vitamer of choice. For those products fortified with folic acid, compliance can be demonstrated by using methods of analysis that are either specific for folic acid or that respond to a broader range of folates.

The most common procedure for the determination of folates in foods is the microbiological assay using *Lactobacillus casei.* Although this assay is highly sensitive, it is time-consuming, labor-intensive, and not consistently reproducible (2). In addition, the growth response of *L. casei* has been reported to vary between folate forms (2–4) and exhibit inhibition or stimulation because of inherent compounds in foods (4). These limitations make the microbiological assay an undesirable routine method for folate analysis in foods.

An alternative to the microbiological assay is liquid chromatography (LC). This technique has been used by numerous researchers to separate folates in standards, blood, tissue, and foods. However, most of the sample preparations fail to extract quantitative (95%) levels of folic acid from foods. The most common extraction involves heating a mixture of sample and

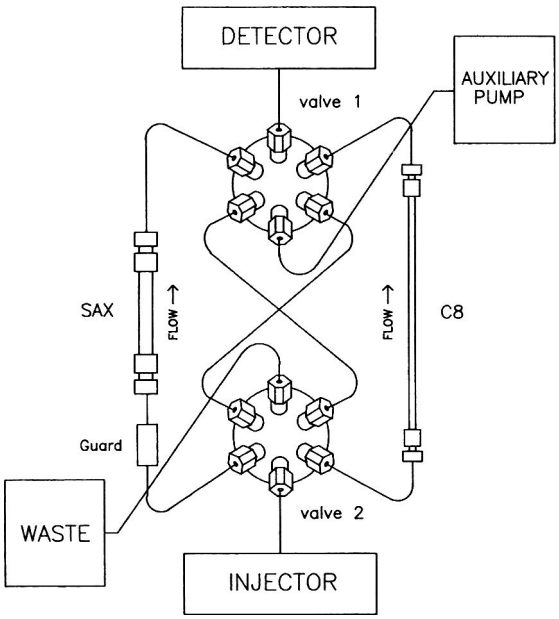

Figure 1. Schematic diagram of the switching valves and their connections to the other LC components. The box labeled "injector" represents the autosampler and binary pumping system of the Hewlett-Packard 1090.

buffered antioxidant solution. This procedure is often used to extract folates from milk and infant formula (5–10). However, Engelhardt (10) reported that less than 80% of added folic acid is recovered from food when a single extraction step is used. In addition, Gregory (4) suggested that 2 extractions are necessary to obtain quantitative recoveries of folic acid in foods. Investigations in this laboratory support these claims. A sample preparation procedure requiring 2 extractions of each sample would significantly increase sample preparation time. As an alternative to the extraction procedure described above, Hoppner and Lampi (11) used the protease papain to extract folic acid from food matrixes. They reported recoveries of folic acid in milk-based and soy-based infant formula to be 96 and 98%, respectively. We have modified and expanded upon this sample preparation procedure to obtain quantitative extraction of folic acid in infant formula and liquid medical nutritional products. The disadvantage of this extraction procedure is that a large number of UV absorbing compounds are formed during enzymatic hydrolysis. Hoppner and Lampi used solid-phase extraction columns to remove potential interferents from the sample preparations before injection into the LC system. We have modified and expanded the procedure of Hoppner and Lampi by altering the sample preparation procedure and by replacing the solid-phase extraction step with an automated procedure using column switching.

This paper describes a method for the determination of folic acid in infant formula and liquid medical nutritional diets. Quantitative extraction of the folic acid is accomplished with enzymatic hydrolysis. LC is used to separate folic acid for quantitation. The sample preparation is initially injected onto a strong anion exchange (SAX) column. Before the elution of the folic acid, the eluant from the SAX column is directed to an octyl bonded-phase column (C_8) by using pneumatic switching valves. After the elution of the folic acid from the SAX column, the SAX column is removed from the flow path by using pneumatic switching valves. An acetonitrile gradient is applied to the C_8 column to elute the folic acid for quantitation. Folic acid is detected by absorption at 345 nm. The mean coefficient of variation (CV) without respect to sample type is 3.6%. The recoveries of added folic acid range from 95.5 to 100.2%. The method has been used to determine folic acid concentrations in infant formulas from various manufacturers. The results of these tests have been consistent with the manufacturer's label claims.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Model 1090 liquid chromatograph equipped with a binary pumping system, 2 L solvent reservoir, UV-vis diode array detector, column switching valve, and column oven (Hewlett-Packard Co.); No. 110 B high pressure pump (Beckman); 6-port pneumatic switching valve.

(b) *LC columns.*—Bio Series SAX analytical column, 8 cm × 6.2 mm, and guard column (Dupont); Zorbax RX (C_8) column, 25 cm × 4.6 mm (Dupont).

Figure 1 illustrates the configuration of the columns and column switching valves. The internal connections between ports of Valves 1 and 2 are not shown, but the various flow paths can be readily deduced. Valve 1 is located within the 1090's column compartment and is controlled by using the column switching commands of the 1090. Valve 2 is located outside the 1090 and is connected to Valve 1 with 0.007 in. id tubing. This valve is connected to 2 switches in the rear of the 1090. These switches are controlled by the contact commands of the 1090. Both the detector and injector are contained within the 1090, and both of the columns are contained within the 1090's column compartment. The auxiliary pump is a Beckman Model 110 B. The auxiliary pump sends mobile phase through the column that is not in-line with the 1090's pumping

Table 1. Column configurations

Configuration No.	Columns in-line between the injector and detector	Columns in-line with the auxiliary pump
Configuration 1	SAX column	C_8 column
Configuration 2	SAX column followed by the C_8 column	None
Configuration 3	C_8 column	SAX column

Table 2. Time table for configuration of columns, mobile phase composition, and flow rate[a]

Step No.	Time since start of run, min	Columns in-line with the injector and detector	Mobile phase composition, %	Flow rate, mL/min
1	0	SAX	100% A	1.5
2	7.9	SAX	100% A	1.5
3	8.0	SAX followed by C_8	100% A	0.8
4	20.0	C_8	100% A	0.8
5	20.1	C_8	84.5% A + 15.5% B	0.8
6	33.0	C_8	84.5% A + 15.5% B	0.8
7	40.0	C_8	100% B	0.8
8	44.0	C_8	100% A	0.8

[a] All of the mobile phase composition and flow rate changes are linear with respect to time. At 45 minutes the run is stopped and the initial conditions restored. The use of SAX columns from current lots necessitate changes in this table. See the *Results and Discussion* section.

system. Three column configurations are used during an analysis. These configurations are listed in Table 1.

Reagents

(a) *Solvents.*—Water, purified using a Millipore purification system, or equivalent; LC grade acetonitrile.

(b) *Folic acid.*—USP Reference Standard.

(c) *Papain.*—Panol 100 (Enzyme Development Co., New York, NY)

(d) *Bacterial protease.*—Enzeco bacterial protease derived from *B. subtilis* (Enzyme Development Co., New York, NY).

(e) *Ammonium hydroxide solution (0.3%).*—Add 6 mL concentrated ammonium hydroxide (28–30%) to 600 mL water.

(f) Mobile phase.—Prepare mobile phase A by dissolving 6.5 g anhydrous sodium acetate and 12.0 g sodium sulfate in water and diluting this to 4 L with water. Adjust pH of this solution to 5.30 with acetic acid, filter, and degas. Prepare mobile phase B by combining 640 mL mobile phase A and 360 mL acetonitrile. (**Note**: The use of Dupont Bioseries SAX columns from current lots necessitates a change in the mobile phase. See the Results and Discussion section.)

(g) *Standard stock solution.*—Follow all label instructions of the USP reference standard. Accurately weigh ca 0.16 g of USP folic acid reference standard. Transfer the folic acid to a 500 mL volumetric flask and dilute to volume with 0.3% ammonium hydroxide. This solution contains ca 290 mg/L folic acid.

(h) *Standard intermediate solution.*—Dilute 2.0 mL of standard stock solution to 100 mL with mobile phase A. This solution contains ca 5.8 mg/L folic acid.

(i) *Standard working solutions.*—Dilute 3.0 and 1.0 mL of standard intermediate solution to 100 mL with mobile phase A. These solutions contain ca 175 and 58 g/L folic acid, respectively.

(j) *Enzyme solution.*—Mix papain (5% weight to volume) and bacterial protease (5% weight to volume) with water and stir until dissolved. Adjust the pH to 8.4 by using a potassium hydroxide solution. The enzyme solution should be used within 1 h of preparation.

Sample Preparation

Determine sample size by estimating the folic acid content of the sample and choosing a sample size so that the folic acid concentration of the final sample preparation is within the range of the standards. Typically, infant formula sample size is 15 mL, and liquid medical nutritional sample size is 3 or 5 mL. Weigh a 150 mL Erlenmeyer flask. Place the appropriate amount of sample into the 150 mL Erlenmeyer flask, and weigh the flask and sample. Add water to the flask to bring the total volume of the sample preparation to ca 15 mL. Autoclave, at 100°C for 5 min, the Erlenmeyer flask and sample preparation of any sample that has not been heat sterilized. Sample preparations of heat sterilized samples need not be autoclaved. Transfer 10 mL of the enzyme solution to the flask. Place the flask in a 40°C water bath, and shake 1 h at 100 rpm. Remove the flask from the water bath, and weigh the flask and sample preparation. Filter the sample preparation through qualitative, medium-speed filter paper (Whatman 2V, or equivalent). Determine the density of the sample preparation by weighing 10.0 mL of the filtrate. Filter an aliquot of the sample preparation through a 0.2 μm filter before injection into the chromatograph.

Liquid Chromatography

Fill the 2 L solvent reservoir and the solvent reservoir of the auxiliary pump with mobile phase A. Fill the 1090's remaining solvent reservoir with mobile phase B. With the columns in Configuration 1 (Table 1; SAX only), begin pumping 100% mobile phase A at 1.5 mL/min from the 1090's solvent delivery system. Begin pumping mobile phase A from the auxiliary pump at 0.8 mL/min. Set the column oven temperature at 35°C.

To ensure system suitability, the SAX column must be monitored before each set of analyses to ensure that folic acid elutes within the specified time limits. After the initial instrument conditions have been set as described above, inject 250 L of the standard intermediate solution onto the SAX column and record the resulting chromatogram. The folic acid should not begin eluting until 0.5 min after the time of the first column switch (Step 2 in Table 2), and it should finish eluting at least 1 min before the time of the second column switch (Step 4 in Table 2). When comparing the time at which the folic acid fin-

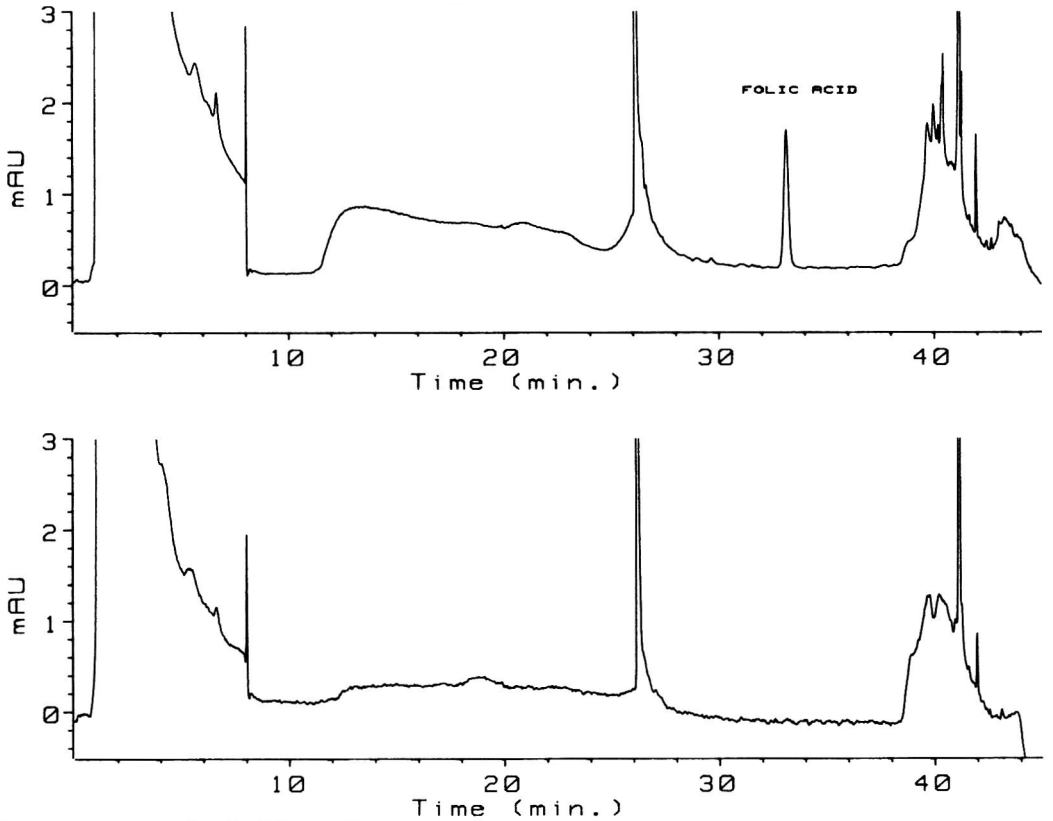

Figure 2. Chromatograms of unfortified milk-based infant formulas with and without added folic acid. Folic acid elutes at 33.5 min.

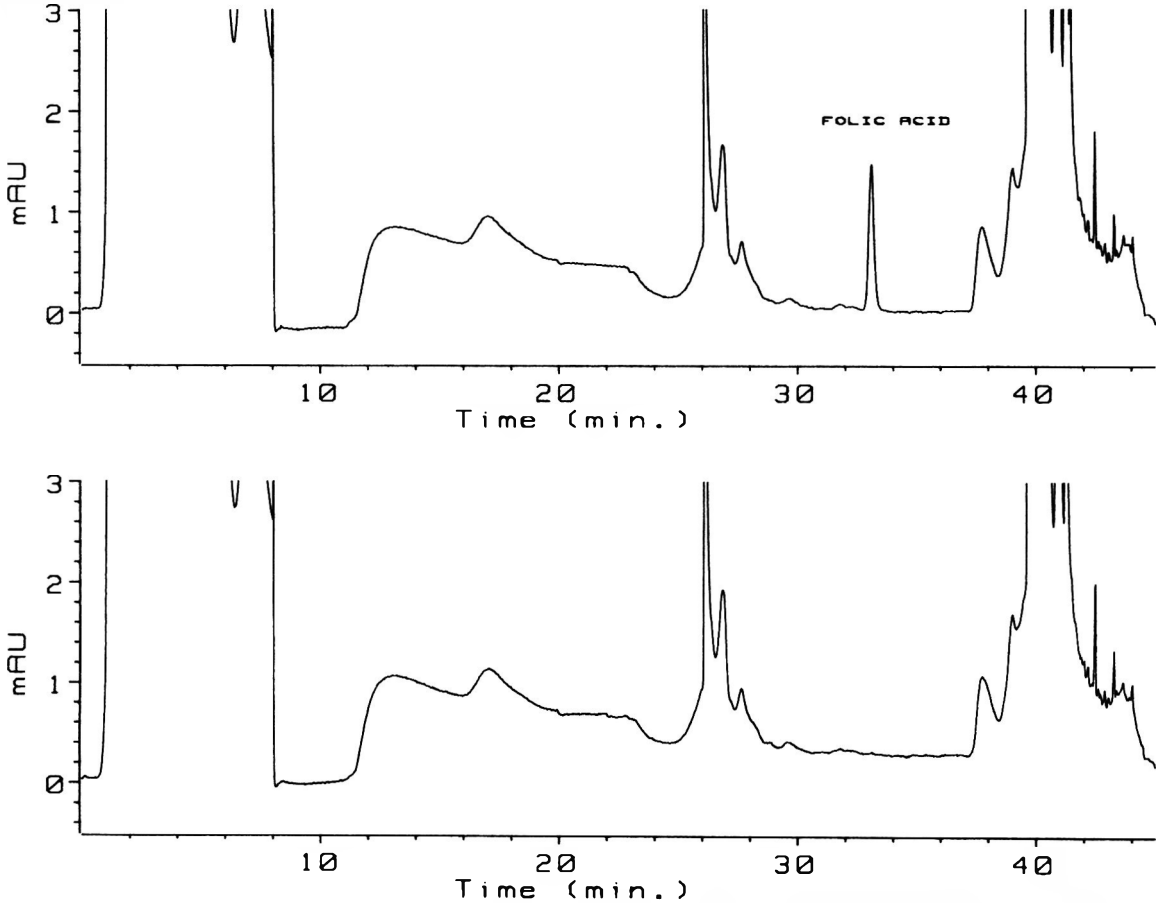

Figure 3. Chromatograms of unfortified soy-based infant formulas with and without added folic acid. Folic acid elutes at 33.5 min.

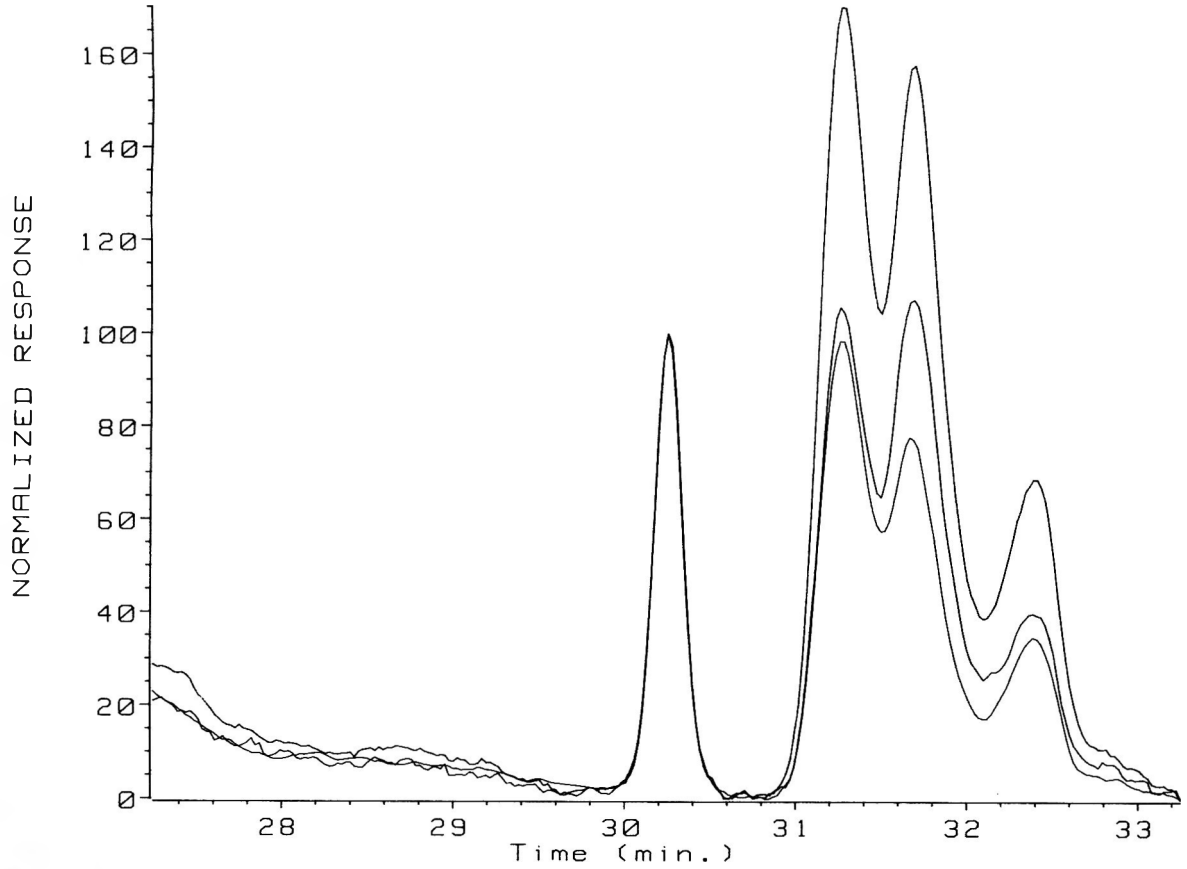

Figure 4. Signal overlay plot of a soy-based infant formula. Signal wavelengths are 295, 345, and 355 nm.

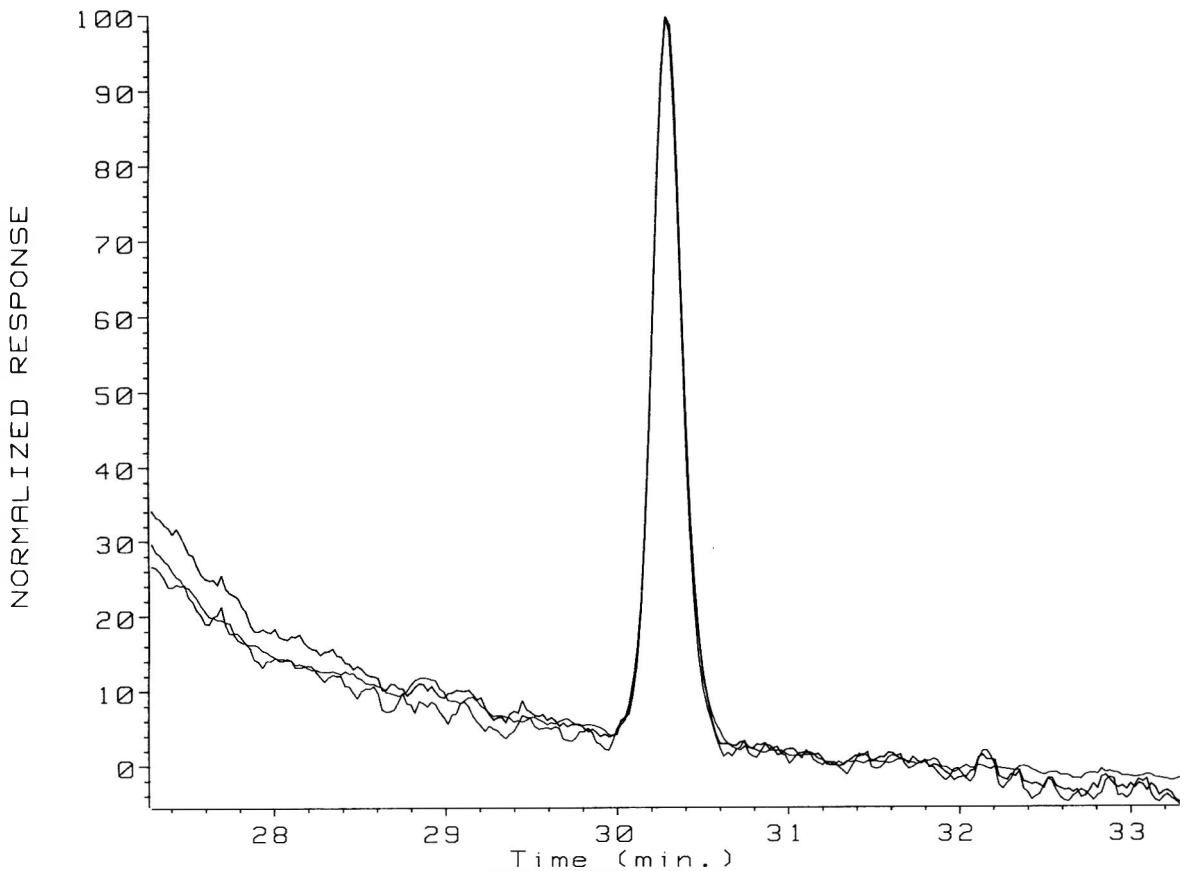

Figure 5. Signal overlay plot of a milk-based infant formula. Signal wavelengths are 295, 345, and 355 nm.

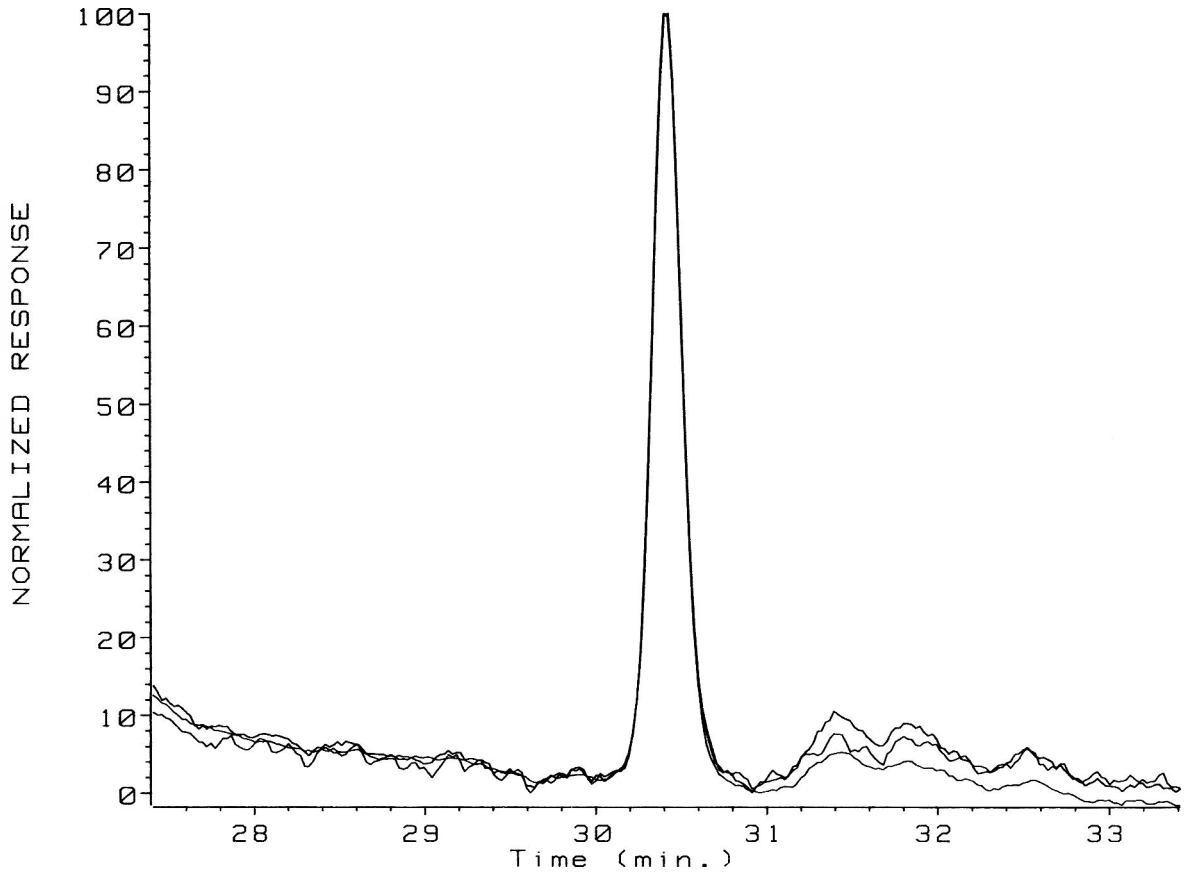

Figure 6. Signal overlay plot of an adult medical nutritional. Signal wavelengths are 295, 345, and 355 nm.

ishes eluting from the SAX column to the time of the second column switch (Step 4 in Table 2), compensation must be made for the discrepancies in the flow rate during the SAX column check, 1.5 mL/min, and the flow rate after the first column switch, 0.8 mL/min. Appropriate time limits for folic acid to elute during a SAX column check, as dictated by the times of Steps 2 and 4 of Table 2, would be 8.4–14.0 min. If the folic acid does not elute within the required time period, either replace the guard column, prepare new mobile phase, replace the SAX column, or adjust the time window.

Begin the analysis by injecting 250 L of standard or sample preparation into the chromatograph. The column configuration, mobile phase, and flow rate changes that occur during the analysis are displayed in Table 2. The times at which Steps 2–5 occur may be altered slightly to suit the individual system. The stop time is 45 min. The detection wavelength is 345 nm with a 10 nm bandwidth. Peak heights are used for quantitation. Folic acid typically elutes between 31 and 33 min (retention times may vary due to column age and the time settings of Steps 2–5 of Table 2). Typical chromatograms of a milk-based and a soy-based infant formula are displayed in Figures 2 and 3.

Table 3. Precision study summary; all values are %RSD

Sample description	Day	Total
Caseine hydrolysate infant formula	1.8	3.4
Soy-based infant formula	1.7	3.8
Whey predominant infant formula	1.6	3.2
Milk-based infant formula	2.7	4.5
Medical nutritional No. 1	3.3	4.2
Medical nutritional No. 2	1.5	2.7
Mean	2.1	3.6

Calculations

Inject one set of working standards, ca 174 and 58 g/L folic acid, before and after each set of samples. The standard curve is a linear plot of the peak heights of the standards vs their concentrations. Determine the folic acid concentration, in grams/liter, of the sample preparations by comparing the peak heights of the folic acid peaks at 345 nm to the standard curve. Determine the folic acid concentration in the original samples by using the following equation:

$$C_o = C_p \times (W_f - W_e)/(W_s - W_e) \times 1/D$$

where C_o is the concentration, in grams/kilogram, of folic acid in the original sample; C_p is the concentration, in grams/liter, of folic acid in the sample preparation; W_f is the weight, in grams, of the Erlenmeyer flask containing the sample prepara-

Table 4. Recoveries of added folic acid

Sample description	No. data points	Mean rec., %
Soy-based infant formula[a]	6	98.3
Milk-based infant formula[a]	6	96.7
Soy-based infant formula	6	97.1
Whey predominant infant formula	6	99.8
Milk-based infant formula	6	100.2
Medical nutritional No. 1	6	95.5
Medical nutritional No. 2	6	95.9

[a] Unfortified formula.

tion after removal from the water bath; W_e is the weight, in grams, of the empty Erlenmeyer flask; W_s is the weight, in grams, of the Erlenmeyer flask with sample; and D is the density of the sample preparation in kilograms/liter.

Results and Discussion

The papain extraction procedure of Hoppner and Lampi has been modified by the addition of a bacterial protease. In our preliminary work, 295 nm was used as the detection wavelength, and the bacterial protease was added to remove interfering peaks in the chromatograms of whey containing samples. Although this procedure was successful in most instances, the chromatograms of some samples displayed interfering peaks even after the addition of the second protease. Consequently, the detection wavelength was changed to 345 nm, a wavelength at which interfering peaks are not observed. However, the bacterial protease was not removed from the sample preparation because the recovery of folic acid from some soy-based samples is improved when this protease is used in conjunction with papain.

The Dupont Bioseries SAX column has been found to be stable under the experimental conditions. During an analysis, the mobile phase passing through this column is always 100% aqueous buffer. This results in the SAX column being especially susceptible to silica dissolution. The Dupont Bioseries SAX column is stabilized to resist dissolution. Bioseries SAX columns from current lots retain folic acid considerably longer than those Bioseries SAX columns used during method development. To maintain the resolution of the folic acid peak without compromising the speed of the analysis, the mobile phase, as well as the acetonitrile concentration, must be altered slightly. The concentration of sodium sulfate in the mobile phase must be increased from 0.022M (12.0 g/4 L) to 0.066M (36.0 g/4 L), and the mobile phase B gradient must be lowered from 15.5% (as listed in Table 2) to 13.9%. Using these conditions, the retention time of folic acid on a Bioseries SAX column from a current lot is very nearly the same as the folic acid retention time on a Bioseries SAX column from an older lot using the conditions specified in *Experimental*. The modification of the acetonitrile gradient maintains the resolution of the folic acid peak in the final chromatogram.

The retention times of strongly retained compounds in soy-based samples gradually declined with repeated injections on all hydrophobic bonded-phase columns (ODS, C_8, Phenyl) that were investigated. These compounds eventually co-eluted with folic acid. Sample preparation modifications failed to correct this problem, and the retention time decline was found to be a function of the volume of aqueous buffer passed over the column and not a function of the number of samples injected. The Dupont RX column (C_8) was found to be much more resistant to this phenomenon than other columns, and separation of folic acid is maintained in soy-based samples after 20 000 column volumes of mobile phase have been passed through the column. Examples of chromatograms of soy-based infant formula can be observed in Figures 3 and 4. The top chromatogram of Figure 3 was obtained using a new C_8 column. Figure 4 is a chromatogram of a similar sample using an aged C_8 column.

Table 5. Analyses of milk-based infant formulas from various manufacturers

Description	Folic acid concn	
	Per weight of sample as fed, µg/kg	As percent of label claim
Manufacturer 1	163	169
Manufacturer 2	71	150
Manufacturer 3	170	175

The process used to determine the precision of the method was an analysis of variance with a balanced 3-stage nested sampling design (12). Six products of dissimilar matrixes were used in this study. The sources of variance investigated were day to day, sample preparation to sample preparation, and injection to injection. The day-to-day CV and the total CV of each product are listed in Table 3. The mean value of the day-to-day CVs is 2.1%, and the mean value of the total CVs is 3.6%. The method detection limit (MDL) was calculated according to U.S. Environmental Protection Agency guidelines (13) and was found to be 6 g/L folic acid, as injected. The method quantitation limit (MQL) was found to be 17 g/L folic acid as injected. These limits are equivalent to 10 and 28 g/kg folic acid, respectively, in ready-to-feed infant formula.

Peak purity evaluations were made by comparing chromatograms of unfortified infant formula to chromatograms of unfortified infant formula with added folic acid and by evaluating signal overlay plots of various products. Figures 2 and 3 display the chromatograms of unfortified samples and unfortified samples with added folic acid of both a milk-based and a soy-based infant formula. No peaks are present in the unfortified sample plots near the time at which folic acid elutes. Signal overlay plots of a soy-based infant formula, a milk-based infant formula, and an adult medical nutritional are shown in Figures 4–6. The chromatograms from 3 signals, 295, 345, and 355 nm, are normalized and overlaid for each sample. There is no indication of folic acid peak distortion in any of these plots. Both the signal overlay plots and the unfortified sample plots indicate that no substances interfere with folic acid at 345 nm.

To assess the accuracy of the method, recoveries of added folic acid were determined from both unfortified and vitamin fortified samples. Folic acid was added to unfortified samples of milk-based and soy-based infant formulas at approximately fortification levels. The mean recoveries were 96.7 and 98.3%,

respectively. Folic acid was added to vitamin fortified samples so that approximately twice the fortification level was obtained. These samples consisted of 2 adult medical nutritionals and 3 infant formulas; milk-based, soy-based, and whey predominant. The mean recoveries of folic acid from these samples ranged from 95.5 to 100.2%. The recovery data of both the unfortified and vitamin fortified samples are summarized in Table 4. These data indicate that the recovery of folic acid by the method is quantitative.

To demonstrate the versatility of the method, folic acid determinations were performed on 3 milk-based infant formulas from various manufacturers. Table 5 contains the results of these determinations. The folic acid concentrations obtained from these analyses are consistent with those expected from the label claims.

In conclusion, the described method provides precise and accurate determinations of folic acid in infant formulas and liquid adult medical nutritional diets. The versatility of the method allows for the effective determination of folic acid in infant formulas from various manufacturers.

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VITAMINS AND OTHER NUTRIENTS

Identification and Quantitation of Panthenol and Pantothenic Acid in Pharmaceutical Preparations by Thin-Layer Chromatography and Densitometry

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A rapid, simple, and specific thin-layer chromatographic (TLC) method has been developed for the estimation of panthenol and pantothenic acid in pharmaceutical preparations containing other vitamins, amino acids, syrups, enzymes, etc. The vitamin is extracted with ethanol (tablets and capsules) or benzyl alcohol (liquid oral preparations) and isolated from other ingredients by TLC on silica gel with isopropanol–water (85 + 15, v/v) as developing solvent. β-Alanine (pantothenate) or β-alanol (panthenol) is liberated by heating for 20 min at 160°C. The liberated amines are visualized by ninhydrin reaction and estimated by spectrodensitometry at 490 nm. Recoveries for panthenol and pantothenic acid were 99.8 ± 2.25% and 100.2 ± 1.7%, respectively.

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Panthenol (PL) and pantothenic acid (PA) as calcium or sodium salts are used in various parenteral or oral preparations of vitamin mixtures. Generally, PL is used in liquid oral and parenteral preparations, and PA salts are used in tablets or capsules. A large number of chemical and microbiological methods are available for the determination of PL and PA (1, 2). The chemical methods are based on acid or alkali hydrolysis of PA or PL followed by colorimetric, fluorometric, or spectrophotometric determination of the vitamins after reaction with suitable chromogenic compounds. However, these chemical methods are difficult to manipulate, because extensive purification procedures must be followed. Also, inadequate separation of interfering chemicals (other vitamins, sugars, amino acids, etc.) as well as poor recovery of PA or PL during purification often lead to erroneous results. Microbiological methods for estimation of PA (1, 2) or PL (1) are very specific and differentiate between the D and L varieties of the vitamins, but these are time-consuming, laborious, and unsuitable for routine analysis. Thus, we tried to develop a simple, specific, and

Table 1. Recovery of panthenol from aqueous solution by benzyl alcohol extraction as determined by proposed method[a]

Aqueous solution		Benzyl alcohol extract		Found[b]	
Concn, mg/mL	Volume, mL	Volume, mL	Concn, mg/mL	Mean ± SD, mg/mL	Rec., %
0.2	5	1	1	0.98 ± 0.01	97.8
0.4	5	2	1	0.998 ± 0.006	99.8
0.6	5	3	1	1.03 ± 0.04	102.6
0.8	5	4	1	1.00 ± 0.01	100.3
1.0	5	5	1	0.98 ± 0.02	98.2

[a] Method for syrup.
[b] Av. of 3 independent determinations.

rapid yet accurate method for the assay of PA and PL in various drug formulations. This paper describes the method we have developed for the assay of PA and PL by thin-layer chromatography (TLC) followed by scanning densitometry.

METHOD

Apparatus

(a) *Densitometer.*—Shimadzu dual-beam TLC scanner, Model CS-930.

(b) *Sample applicator.*—Camag Nanomat II with 1 μL capillary and holder.

(c) *TLC plates.*—Silica gel 60 precoated aluminum-backed sheets, 10 × 10 cm.

(d) *Spray gun.*—Glass sprayer fitted with air pump (oil-free) or compressed air can. Hand pumps are not suitable.

(e) *Hot air oven.*—With air circulator, set at 160 and 105°C.

Reagents

(a) *Standard solutions.*—(±)-Pantothenyl alcohol (USP Reference Standard) and calcium D-pantothenate (1 + 2) (USP Reference Standard), 1 mg/mL in water–ethanol (1 + 4, v/v).

(b) *Ethanol.*—Distilled, dehydrated ethanol, USP grade.

(c) *Developing solvent.*—*(1)* Chloroform, use as is. *(2)* Isopropanol–water (85 + 15, v/v).

(d) *Chromogenic reagent.*—Ninhydrin (E. Merck, Darmstadt, Germany). Dissolve 300 mg in 100 mL *n*-butanol previously saturated with water. Before spraying, add 0.1 mL glacial acetic acid/10 mL of the solution.

(e) *Solvent.*—All solvents used were LiChrosolv, E. Merck grade.

Preparation of Sample

(a) *Tablets.*—Select 20 intact tablets, weigh accurately, and grind to smooth powder with mortar and pestle. Accurately weigh amount of powder equivalent to ca 10 mg PL or PA (salt) and quantitatively transfer to 10 mL volumetric flask; add 2 mL water, shake thoroughly on vortex mixer 2 min, add 5 mL ethanol, mix, and dilute to volume with ethanol. Mix again and let excipients settle. Use clear supernatant for TLC.

(b) *Capsules.*—Accurately weigh contents of 20 capsules and mix thoroughly with mortar and pestle. Accurately weigh amount of powder equivalent to ca 10 mg PL or PA (salt) and treat as for tablets.

(c) *Liquid preparations other than syrups.*—Dilute pharmaceutical preparations like vitamin injections and pediatric drops with ethanol to produce 1 mg PL/mL. If any precipitate appears, remove it by centrifugation (3000 × *g*), and use clear supernatant alcoholic solutions for TLC. For liquids containing <2 mg of the vitamin/mL (<1 mg/mL for parenteral preparations), treat as syrups (described below).

(d) *Syrups.*—Transfer amount equivalent to 1 mg PL to test tube. Add ca 0.9 g ammonium sulfate powder/mL sample. Shake, add 1 mL benzyl alcohol, and shake on vortex mixer for

Table 2. Effect of time on degradation of panthenol and pantothenic acid on silica gel TLC plates at 160°C

	Integrated area value[a]	
Time, min	Panthenol	Pantothenic acid
10	27415 ± 1924	19581 ± 1372
15	37672 ± 433	26916 ± 319
20	37581 ± 459	26743 ± 339
40	37706 ± 310	26938 ± 221

[a] Av. of 4 determinations.

Table 3. Stability of ninhydrin color complex of panthenol and pantothenic acid on silica gel TLC plates determined by scanning densitometry at 490 nm

	Integrated area value	
Time, min	Panthenol	Pantothenic acid
5	69062	38726
30	69240	38772
60	68717	38642
90	68718	37684
120	68869	38953
240	68767	38835

Table 4. Recovery of D-panthenol and calcium D-pantothenate added to commercial vitamin B complex or multivitamin preparations and determined by proposed method

Sample No.[a]	Declared mean ± SD, mg[b]	Added, mg	Total mean ± SD, mg	Found mean ± SD, mg	Rec., %
		Panthenol			
1		10	9.93 ± 0.17	9.93 ± 0.17	99.3
2		5	5.08 ± 0.12	5.08 ± 0.12	101.6
3	2.18 ± 0.03	2	4.19 ± 0.06	2.00 ± 0.02	100.3
4	1.95 ± 0.03	2	3.98 ± 0.09	2.03 ± 0.06	101.4
5	5.09 ± 0.03	5	9.90 ± 0.05	4.81 ± 0.03	96.2
Overall rec.					99.8 ± 2.2
		Calcium pantothenate			
6	0	5	5.15 ± 0.10	5.15 ± 0.10	103.0
7	1.07 ± 0.04	1	2.06 ± 0.08	1.00 ± 0.06	100.0
8	5.05 ± 0.13	5	9.98 ± 0.05	4.93 ± 0.12	98.5
9	4.67 ± 0.06	5	9.73 ± 0.18	5.06 ± 0.17	101.2
10	12.54 ± 0.20	12.5	24.98 ± 0.25	12.44 ± 0.06	99.5
11	6.64 ± 0.12	5	11.58 ± 0.21	4.94 ± 0.10	98.8
Overall rec.					100.2 ± 1.7

[a] Sample composition: (1) Vitamin B1, 50 mg; B6, 50 mg; B12, 0.5 mg (per mL injection). (2) Vitamin B1, 33 mg; B6, 33 mg; B12, 0.33 mg (per mL injection). (3) D-Panthenol, 2 mg; vitamin B1, 10 mg; B2 5-phosphate, 2 mg; niacinamide, 50 mg (per mL injection). (4) D-Panthenol, 2 mg; vitamin A palmitate, 3000 IU; D2, 300 IU; B1, 1 mg; B6, 1 mg; B2 5-phosphate, 0.5 mg; niacinamide, 5 mg; L-lysine HCl, 25 mg; color tartrazine (per mL drop). (5) D-Panthenol, 5 mg; vitamin B1, 4.5 mg; B2, 5.0 mg; B6, 1.5 mg; niacinamide, 50 mg; color Ponceau 4R (per 5 mL liquid syrup). (6) Vitamin B1, 10 mg; B6, 3 mg; B12, 0.015 mg; glutamic acid, 100 mg (tablet). (7) Calcium D-pantothenate, 1 mg; vitamin A, 2500 IU; D, 200 IU; B1, 2 mg; B2, 2 mg; B6, 0.5 mg; C, 50 mg; niacinamide, 25 mg; folic acid, 0.2 mg; color erythrosin (sugar-coated tablets). (8) Calcium D-pantothenate, 5 mg; vitamin B1, 5 mg; B2, 5 mg; B6, 2 mg; niacinamide, 50 mg (capsules). (9) Calcium D-pantothenate, 5 mg; vitamin B1, 5 mg; B2, 5 mg; B6, 2 mg; niacinamide, 50 mg (tablet). (10) Calcium D-pantothenate, 12.5 mg; vitamin B1, 10 mg; B2, 10 mg; B6, 3 mg; C, 150 mg; B12, 5 μg; niacinamide, 50 mg; folic acid, 1 mg (capsule). (11) Calcium D-pantothenate, 5 mg; thiamin propyldisulfide, 4.5 mg; vitamin B2, 5 mg; B6, 1.5 mg; B12, 5 μg; vitamin C, 75 mg; niacinamide, 45 mg; folic acid, 1 mg (tablet).

[b] Av. of 4 independent determinations.

2 min. Allow benzyl alcohol layer to separate. Use clear top organic layer for TLC. For syrups containing >1 mg PL/mL, dilute with water to produce 1 mg PL/mL before adding ammonium sulfate.

Thin-Layer Chromatography

Apply 1 μL each of sample or standard extract as compact spots 1 cm apart and 1 cm from bottom or edge of TLC sheet. For benzyl alcohol extracts, develop plate first with solvent *(1)* to full height. Dry briefly in air (10 min), and develop again in solvent *(2)* to full height. For ethanol extracts, omit first development. After development, heat plate in hot-air oven at 160°C for 20 min, cool, spray uniformly *(3)* with ninhydrin reagent until slightly moist, and heat again at 105°C for 10 min. Scan spots at 490 nm in zigzag mode with linearlizer off. Set other parameters as given in operation manual of scanner.

Method Evaluation

Optimum heating time for quantitative degradation of PL and PA was determined after TLC of respective standards applied (1 μL) as series of spots on TLC sheet. After development, TLC sheet was cut in direction of development and resulting strips were heated at 160°C for different lengths of time. Color was developed and evaluated, as described above.

Relation of integrated area value to amount of PA or PL chromatographed was determined by applying 1 μL each of respective standard solution (0.5–8 mg/mL) and evaluating as described above.

To determine efficiency of benzyl alcohol extraction of PL, different concentrations (0.2–1 mg/mL) of vitamin in water were prepared and assayed by method proposed for syrup.

Stability of ninhydrin complex was evaluated by scanning colored spots 4 times at 1 h intervals without taking plate out of scanner.

Results and Discussion

When heated at 160°C, PL or PA produce β-alanol or β-alanine, respectively, which reacts with ninhydrin at 105°C in an acidic pH to produce intensively colored compounds (1, 4). This reaction is used to detect PA and PL on TLC plates (4). In our experience, recovery (extractions with ethanol) of these colored compounds from TLC plates is poor; therefore, spectrophotometric determination of PA and PL after visualization with ninhydrin is not possible. We used reflection densitometry for in situ determination of these vitamins after TLC. Relation of the integrated area value to amount of PL and PA was determined. The regression analysis shows excellent linear relation-

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ship within a concentration range of 0.5–8 μg for both vitamins (r = 0.9996 for PL and 0.9994 for PA).

Benzyl alcohol quantitatively extracts PL from aqueous solution saturated with ammonium sulfate within the concentration range of 0.2–1 mg/mL (Table 1).

Time required for quantitative degradation of PL and PA at 160°C was 15 min (Table 2), and the color produced with ninhydrin was stable for at least 4 h (Table 3). Thus, we used a heating time of 20 min at 160°C for degradation of the vitamins, and scanned the chromatogram on the same day.

Table 4 shows recoveries of PL and PA added to different commonly available samples. The average recoveries were 99.8% for PL and 100.2% for PA.

Quantitative analysis by scanning densitometry is an ideal process for rapid estimation when the number of samples is large (5). We have successfully used densitometry for estimation of various pharmaceutical products (5–7) and have already assayed more than 500 samples of different dosage forms containing PA or PL by the proposed method without any difficulty. Because the assay is done after the vitamins are isolated by TLC, interferences from other vitamins, coloring materials excipients, etc., are not encountered. Also, natural amino acids have lower R_f values than PA or PL in the proposed TLC system and do not interfere with identification or assay of these vitamins. In addition, amino acids (like lysine, glutamic acid, and methionine) commonly added to vitamin tonics may easily be identified and assayed without extra cost or labor, as they become well-separated and are readily detected under the experimental conditions described.

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TECHNICAL COMMUNICATIONS

Liquid Chromatographic Assay for Naphazoline and Antazoline in Ophthalmic Preparations

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A liquid chromatographic (LC) analysis has been developed for determining naphazoline and antazoline in ophthalmic preparations. The LC system is designed so that a variety of ophthalmic solutions containing naphazoline and antazoline can be analyzed with precision.

Naphazoline, an alpha adrenergic agent, is usually combined with other drugs in nasal and ophthalmic decongestant solutions at concentrations ranging from 0.012 to 1.0%. A frequent combination is 0.05% antazoline and 0.5% naphazoline.

Antazoline and naphazoline are bases with very similar pK_a values, 10.1 and 10.9, respectively (1), and with very similar chemical and structural properties (Figure 1). Thus, it is impossible to quantitate both drugs in mixtures by functional group methods, especially if the drugs are combined in a 10:1 proportion (2).

Anhydrous titration is a quantitative analytical method for this type of compound as pure drug (3, 4). The purity of antazoline preparations may be controlled by gravimetric (5), colorimetric, or UV absorption methods (6). Pure naphazoline may be quantitated by colorimetry (7) or UV absorbance (3), and naphazoline in nasal solutions may be quantitated by liquid chromatography (LC) (3).

The drugs have been determined in naphazoline and antihistamine preparations by liquid-liquid column chromatography after quantitative determination by absorptiometry (8). The resulting method is complex and time-consuming.

In this study, a high resolution LC method has been developed for naphazoline preparations and applied to the analysis of ophthalmic preparations.

Experimental

Reagents

Methanol, citric acid, sodium citrate, and perchloric acid, LC grade. Naphazoline hydrochloride, antazoline phosphate, and tetrahydrozoline hydrochloride, containing ≥99.0%. Commercial ocular decongestants, containing 500.0 mg% antazoline phosphate and 50.0 mg% naphazoline hydrochloride.

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Apparatus

Liquid chromatograph (pump, Waters Model 510; variable wavelength detector, Model 441). Analytical wavelength, 254 nm; flow rate, 2.0 mL/min; injection volume, 20 µL. Column, microparticulate octadecylsilane (Waters-Bondapak C-18). The experiments were done at room temperature.

Preparation of Mobile Phase and Standard Solutions

(a) *Mobile phase.*—To 700 mL water, add 6 g sodium citrate dihydrate and 4 g anhydrous citric acid, mix until dissolved, and add 7 mL perchloric acid. Determine pH and adjust to 2.2 ± 0.2 by further addition of perchloric acid. Add 300 mL methanol, mix thoroughly, filter through 0.45 µm filter, and deaerate 10 min.

(b) *Tetrahydrozoline internal standard solution.*—Prepare solution by dissolving 50 mg tetrahydrozoline hydrochloride in distilled water and diluting to 100 mL. Place 10 mL of this solution into 100 mL volumetric flask and dilute to volume with distilled water. Use this solution to measure retention time.

(c) *Naphazoline standard.*—Accurately weigh ca 50 mg naphazoline hydrochloride into 100 mL volumetric flask, dissolve in distilled water, and dilute to volume. Pipet 10 mL of this solution into 100 mL volumetric flask and dilute to volume with distilled water.

(d) *Antazoline standard.*—Accurately weigh ca 50 mg antazoline phosphate into 100 mL volumetric flask, dissolve in distilled water, and dilute to volume. Pipet 10 mL of this solution into 100 mL volumetric flask and dilute to volume with water.

Preparation of Calibration Solution

Pipet drugs and internal standard into separate 100 mL volumetric flasks and dilute to obtain concentrations ranging from 2.0 to 8.0 mg% drugs and 5 mg% internal standard.

Sample Preparation

(a) *Naphazoline hydrochloride.*—Pipet 4.0 mL commercial ocular decongestant into 100 mL volumetric flask, add

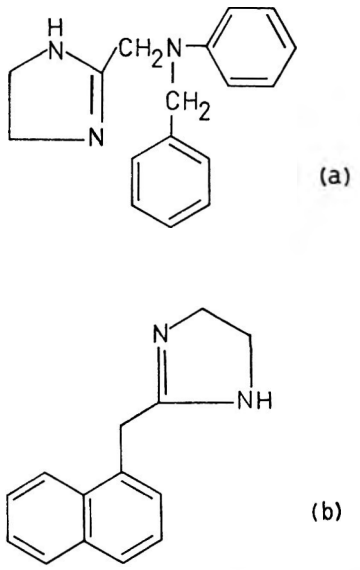

Figure 1. Chemical structures of antazoline (a) and naphazoline (b).

10 mL tetrahydrozoline internal standard solution, and dilute to volume with distilled water.

(b) *Antazoline phosphate.*—Pipet 1.0 mL commercial ocular decongestant into 100 mL volumetric flask, add 10 mL tetrahydrozoline internal standard solution, and dilute to volume with distilled water.

Calculate content of each drug according to calibration curve.

Results and Discussion

The retention times were obtained from the chromatograms, at the optimal experimental conditions obtained (Figure 2,

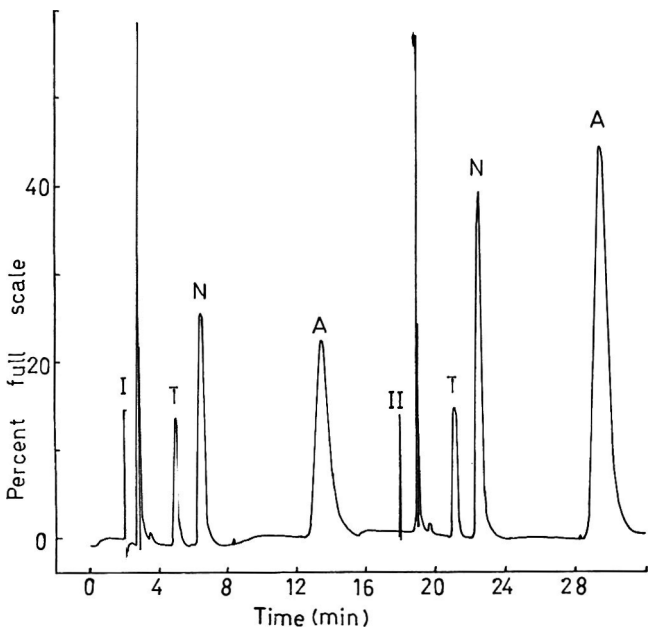

Figure 2. Chromatogram of naphazoline (N)–antazoline (A) mixture. Internal standard: tetrahydrozoline (T). I and II are duplicate injections.

Table 1. Retention time of antazoline phosphate, naphazoline hydrochloride, and tetrahydrozoline hydrochloride

Drug	Retention time, min
Antazoline phosphate	11
Naphazoline hydrochloride	4
Tetrahydrozoline hydrochloride	3

Table 2. LC calibration curve for pure naphazoline hydrochloride[a]

Concn, mg%	Peak area ratio, naphazoline/tetrahydrozoline
2.00	2.332
3.00	3.523
4.00	4.715
5.00	5.906
6.00	7.097

[a] Internal standard, tetrahydrozoline hydrochloride. Linear equation: A = 1.1913 C − 0.0506 (r = 0.9999).

Table 3. LC calibration curve for pure antazoline phosphate[a]

Concn, mg%	Peak area ratio, antazoline/tetrahydrozoline
2.00	5.105
4.00	10.352
5.00	12.976
6.00	15.599
8.00	20.846

[a] Internal standard, tetrahydrozoline hydrochloride. Linear equation: A = 2.6235 C − 0.1421 (r = 0.9999).

Table 4. Recoveries of antazoline phosphate and naphazoline hydrochloride by LC

	Rec., %	
Sample[a]	Antazoline phosphate	Naphazoline hydrochloride
1	99.0	99.4
2	99.9	99.0
3	98.4	98.1
4	99.0	99.5
5	99.8	99.0
$\bar{X}$, %	99.2	99.0
SD, %	0.63	0.55

[a] Oftalirio, Saval Laboratories, Santiago-Chile. Declared amounts: antazoline phosphate, 500.0 mg%; naphazoline hydrochloride, 50.0 mg%.

Table 5. Percentages of labeled amounts of antazoline phosphate and naphazoline hydrochloride determined by LC

Sample[a]	Run	Antazoline phosphate, %	Naphazoline hydrochloride, %
I	1	101.3	100.2
	2	100.5	100.6
II	1	99.9	99.4
	2	98.4	102.7
III	1	101.5	100.7
	2	101.7	101.7
IV	1	98.8	97.2
	2	100.5	100.6
V	1	100.8	98.1
	2	100.5	100.6
VI	1	102.7	99.5
	2	100.5	100.6
$\bar{X}$, %		100.6	100.2
SD, %		1.2	1.5

[a] Oftalirio, Saval Laboratories, Santiago-Chile. Declared amounts: antazoline phosphate, 500.0 mg%; naphazoline hydrochloride, 50.0 mg%.

Table 1). The internal standard method gave linear calibration curves for naphazoline at concentrations ranging from 0.02 to 0.06 mg/mL, with a 0.9999 correlation coefficient (Table 2), and for antazoline at concentrations ranging from 0.02 to 0.08 mg/mL, with a 0.9999 correlation coefficient (Table 3). Mean recoveries, shown in Table 4, were 99.2 and 99.0% for antazoline and naphazoline, respectively.

The percentages of label declaration for commercial ocular decongestant preparations were 100.6% (standard deviation, 1.2%) for antazoline and 100.2% (standard deviation, 1.5%) for naphazoline (Table 5).

The results indicate that this method may be easily applied to quantitative determination of antazoline phosphate and naphazoline hydrochloride in ocular decongestant preparations containing the 2 drugs in a 10:1 ratio.

Because of its sensitivity, precision, speed, and simplicity, this method is superior to the method previously used for this drug combination (8). Moreover, it can adequately resolve the naphazoline–antazoline mixture in ophthalmic preparations.

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TECHNICAL COMMUNICATIONS

Determination of Milk Proteins by Capillary Electrophoresis

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The potential utility of capillary electrophoresis (CE) for routine determination of milk protein is established. Proteins in cow's milk can be determined by CE in 10 min with high separation efficiency. The major protein components of milk are well-separated and identified. Separations of milk proteins are achieved reliably and reproducibly in an untreated fused-silica column of 21 μm id × 23–25 cm. Fresh homogenized, low-fat, and nonfat milk show almost identical contents of each protein species; dry milk has a substantially reduced amount of whey proteins, especially α-lactalbumin. Extensive degradation of whey proteins is evident from a reconstituted dry milk, which may be used to differentiate dry from fresh milk. By using the ratio of β-casein to α-lactalbumin, the adulteration of fresh milk with 25% or more of dry milk could easily be detected.

Cow's milk contains about 3.2% protein regardless of its fat content. Protein in cow's milk is a complex mixture; 80% of protein is casein, and the remainder is whey proteins, with 12% β-lactoglobulin and 5% α-lactalbumin. Casein is a phosphoprotein and occurs in 4 distinct types, α-, β-, τ-, and k-caseins, respectively, representing about 50, 35, 5, and 15% of this protein (1). In milk, casein molecules are assembled in aggregates of micelles. Whey proteins are soluble and have been well-characterized (2, 3). Milk proteins have been separated by gel electrophoresis (4), ion-exchange chromatography (5), and gel permeation chromatography (6). Total protein content in milk can be determined by a variety of methods, including Kjeldahl (7), dye-binding colorimetry (8), and infrared spectrophotometry (9). Because most of the separation techniques and assay procedures for milk proteins are too cumbersome for routine use in a dairy plant, cost of milk has seldom been based on its protein quality or content.

Recent developments with capillary electrophoresis (CE) represent an important technical advance in analytical zone electrophoresis (10–14). Analytes in the sample are separated electrophoretically into discrete zones in an open capillary column without significant diffusion. The feasibility of performing a routine protein determination by CE in a fused-silica column has been demonstrated (15). We have also elucidated the advantages of using capillaries with inside diameters of 25 μm or less for the rapid determination of protein in complex mixtures such as human serum (16). This report is an attempt to establish the feasibility of the rapid determination of milk proteins by CE. The application of the CE technique to the detection of adulteration of fresh milk with cheaper powdered milk is illustrated.

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Experimental

CE Procedures

A P/ACE 2100 automated CE system (Beckman Instruments, Inc., Palo Alto, CA) was used with P/ACE system software controlled by an IBM PS/2 Model 55 SX. Postrun data analysis was performed on System Gold software (Beckman Instruments, Inc., Fullerton, CA). Capillary columns 23 or 25 cm long (16.5 or 18.5 cm to detector window) × 21 μm id (Polymicro Technologies, Phoenix, AZ) were assembled in the P/ACE cartridge format (100 × 200 μm aperture). The P/ACE system used on-line detection at 200 nm. During electrophoresis, the capillary was maintained at ambient temperature (usually 23°C) with circulating coolant surrounding the capillary. Diluted samples were introduced by pressure injection for 15–25 s, and electrophoresis was performed at the voltage indicated on the electropherograms. Between runs, the capillary was sequentially washed with 2 column volumes of 1.0N sodium hydroxide and water (0.3 min high pressure rinsing each), followed by reconditioning with 5–10 volumes of run buffer (2.5–3 min high pressure rinsing).

Buffer and Sample Preparations

Phosphate buffers at pH 6.0–9.0 were prepared by mixing the appropriate amount of 0.5M mono- and disodium phosphate. To prevent the aggregation of the casein proteins, 4M urea was added, and all buffers were filtered through a 0.45 μm filter before use. Protein standards, such as α-, β-, and k-caseins, α-lactalbumin, and β-lactoglobulins A and B, were obtained from Sigma Biochemicals (St. Louis, MO). Fresh milk and nonfat dry milk samples were purchased from a local supermarket. Protein standards were dissolved in buffer containing 75mM sodium chloride, 20mM potassium phosphate, and 0.01% sodium azide, pH 7.0 (PBS). The concentration of each protein was ca 1.0 mg/mL, and the minimum detection level at 200 nm for a protein is ca 50 μg/mL. Powdered nonfat milk

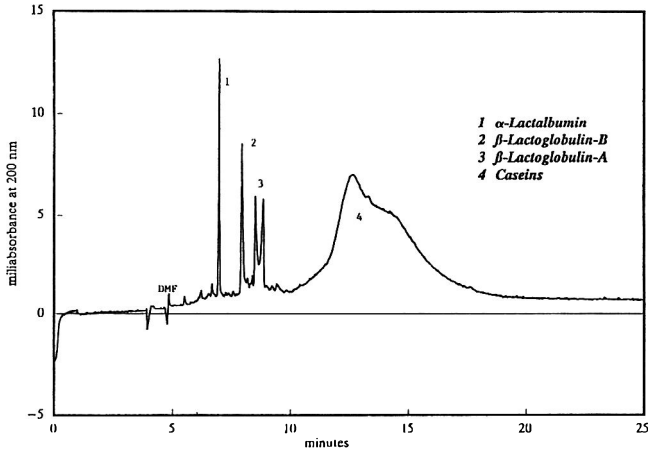

Figure 1. Electropherogram of nonfat milk at pH 8.0, 25 cm × 21 μm column, 10 kV, 66 μA, 05M phosphate pH 6.0.

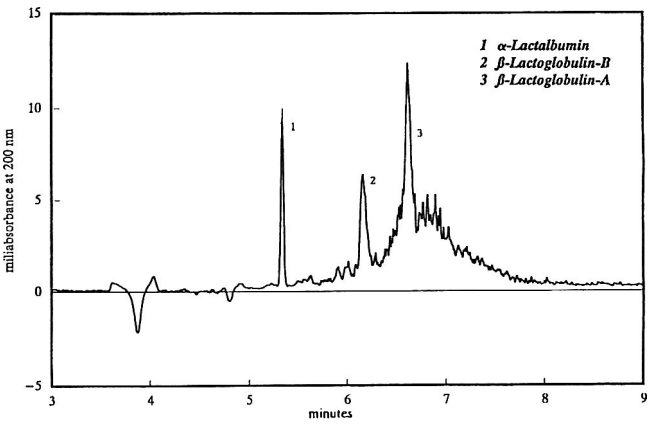

Figure 2C. Electropherogram of homogenized milk, 25 cm × 21 μm column, 10 kV, 45 μA, 05M phosphate pH 6.0.

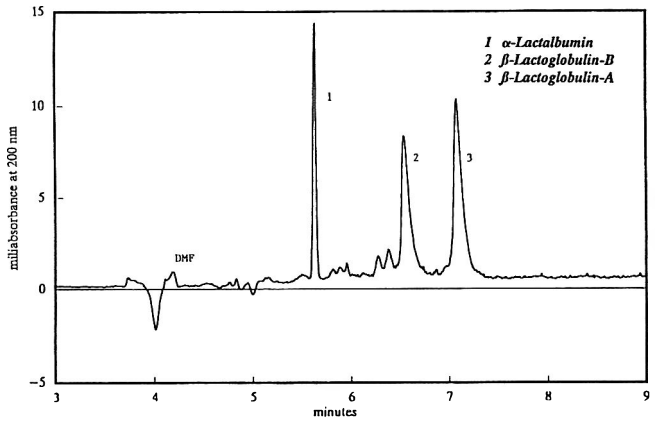

Figure 2A. Electropherogram of nonfat milk, 25 cm × 21 μm column, 10 kV, 45 μA, 05M phosphate pH 6.0.

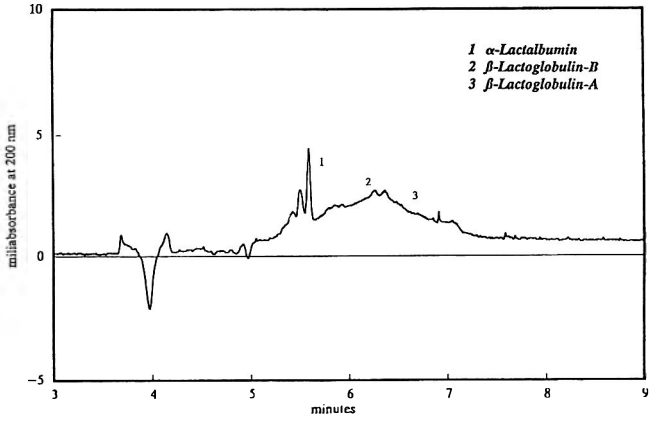

Figure 2D. Electropherogram of reconstituted powder milk, 25 cm × 21 μm column, 10 kV, 45 μA, 05M phosphate pH 6.0.

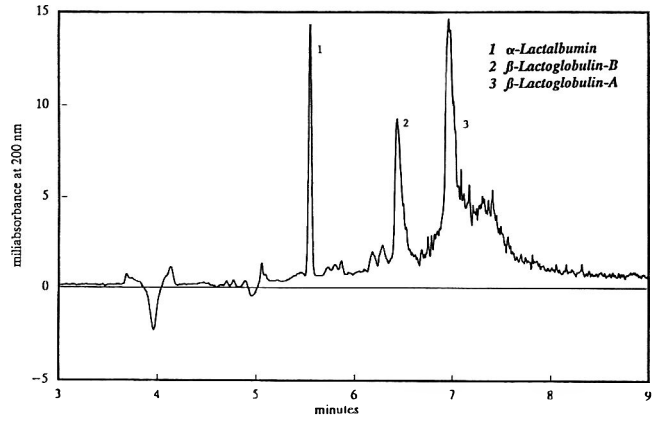

Figure 2B. Electropherogram of low-fat milk, 25 cm × 21 μm column, 10 kV, 45 μA, 05M phosphate pH 6.0.

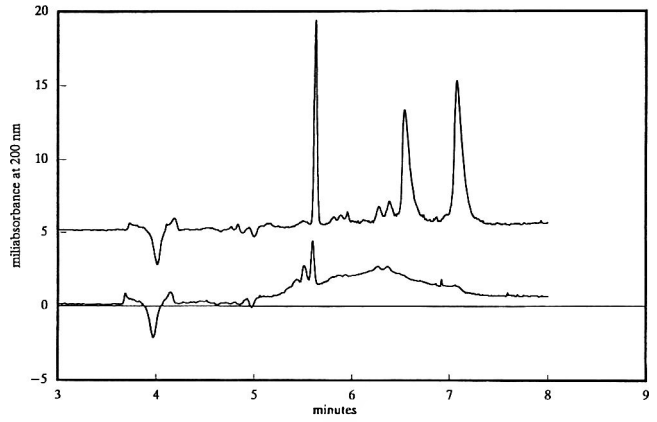

Figure 2E. Electropherogram of nonfat (upper) and powdered milk (lower), 25 cm × 21 μm column, 0.5M phosphate pH 6.0.

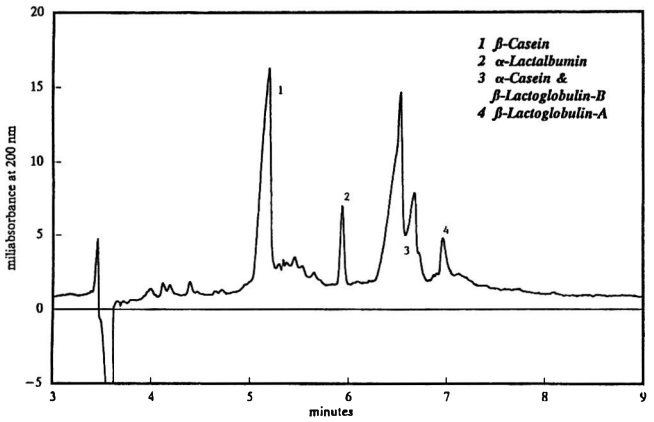

Figure 3A Electropherogram of fresh nonfat milk, 23 cm × 21 μm column, 10 kV, 51 μA, 0.5M sodium phosphate, 4.0M urea pH 7.0.

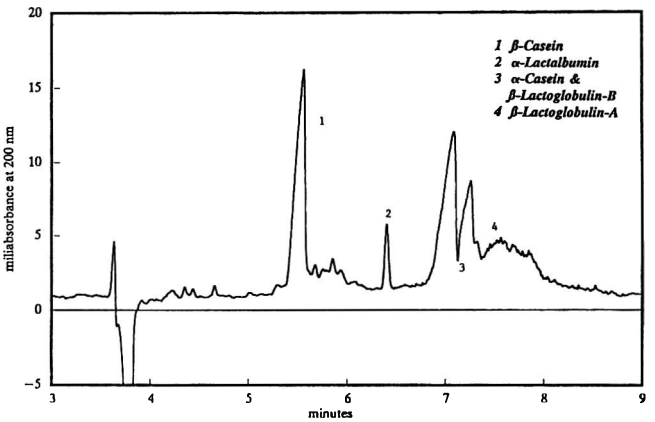

Figure 3C. Electropherogram of fresh homogenized milk, 23 cm × 21 μm column, 10 kV, 51 μA, 0.5M sodium phosphate, 4.0M urea pH 7.0.

was dissolved in water according to the procedure recommended by the manufacturer. All milk samples were diluted at 1 + 5 in PBS. Dimethylformamide (DMF) at 0.01% (v/v) was added to the sample diluent as an electroosmotic flow marker.

Results and Discussion

Caseins constitute the major group of milk proteins. They are phosphoproteins that consist of subspecies α-, β-, k-, and τ-casein and are assembled in an aggregate of micelles. Attempts to separate the caseins by CE in phosphate-based buffers at pH values between 7 and 9 show a single unresolved broad peak (Figure 1) with phosphate buffer at pH 8.0. Whey proteins are well-resolved; α-lactalbumin and β-lactoglobulins B and A migrate at 6.8, 8.1, and 8.6 min, respectively. The peak that occurs immediately after β-lactoglobulin A at 8.9 min is a degradation product of β-lactoglobulin A (with pure β-lactoglobulin A, both peaks are present when the running buffer pH is 8.0 or above, whereas at a lower buffer pH such as 6.0, β-lactoglobulin A migrates as a single species). Determination of milk proteins with phosphate buffer at pH 6.0 indicates that all whey proteins, such as α-lactalbumin and β-lactoglobulins B and A, are well-separated and occur in similar quantities in fresh milk, regardless of their fat contents. All caseins, which make up 80% of the milk proteins, were not observed in the electropherogram after 30 min. Figures 2A, 2B, and 2C show the presence of whey proteins in fresh nonfat, low-fat, and whole milk, respectively. α-Lactalbumin migrates at about 5.3 min, followed by β-lactoglobulins B and A at 6.8 and 7.5 min. The broad peak underneath β-lactoglobulin A is presumably a minor protein fraction associated with lipid. For reconstituted milk, however, the whey proteins appear between 5.6 and 7.3 min as rather broad peaks as shown in Figure 2D. Figure 2E exhibits the electropherograms of both nonfat and reconstituted milks for comparison. The absence of lipid-associated minor proteins in both types of milk is evident from Figure 2E. Whey proteins are clearly exhibited in nonfat milk, whereas the protein in the reconstituted milk appears to be broad and ill-defined, indicating extensive degradation (2, 3).

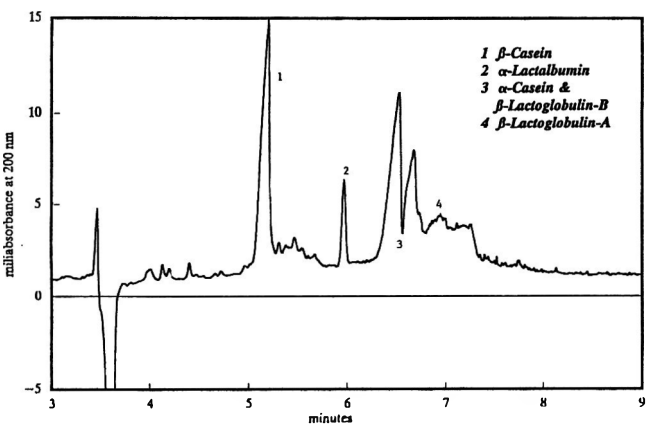

Figure 3B. Electropherogram of fresh low-fat milk, 23 cm × 23 μm column, 10 kV, 52 μA, 0.5M sodium phosphate, 4.0M urea pH 7.0.

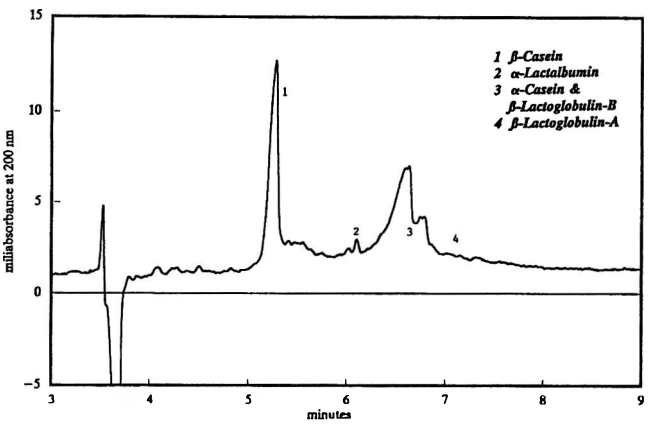

Figure 3D. Electropherogram of reconstituted powder milk, 23 cm × 21 μm column, 10 kV, 52 μA, 0.5M sodium phosphate, 4.0M urea pH 7.0.

Table 1. Quantitation of each protein fraction in nonfat and reconstituted powder milk in 3 runs

Protein faction	Peak area	RSD, %	Peak height	RSD, %
		Nonfat		
β-Casein	1.28	5.48	12.80	5.63
α-Lactalbumin	0.22	2.24	4.95	4.65
α-Casein 1	1.37	4.76	10.20	3.82
α-Casein 2	0.50	7.59	5.18	4.63
β-Lactoglobulin A	0.17	2.89	2.45	8.57
β-Casein/α-lactalbumin ratio	5.73	NA[a]	2.59	NA
		Dry milk 25% and nonfat 75%		
β-Casein	1.30	5.41	13.20	5.07
α-Lactalbumin	0.17	3.25	3.81	3.94
α-Casein 1	1.31	5.17	8.91	3.93
α-Casein 2	0.42	8.41	8.90	4.94
β-Lactoglobulin A	0.11	2.89	1.43	14.01
β-Casein/α-lactalbumin ratio	7.74	NA	3.46	NA
		Dry milk 50% and nonfat 50%		
β-Casein	1.55	5.97	13.76	5.16
α-Lactalbumin	0.15	3.09	3.29	5.17
α-Casein 1	1.57	6.62	9.38	4.90
α-Casein 2	0.43	10.87	4.75	8.84
β-Lactoglobulin A	—[b]	NA	—	NA
β-Casein/α-lactalbumin ratio	10.16	NA	4.18	NA
		Dry milk 75% and nonfat 25%		
β-Casein	1.38	8.98	12.70	7.01
α-Lactalbumin	0.11	6.18	2.20	8.63
α-Casein 1	1.23	9.59	7.26	5.51
α-Casein 2	0.44	10.37	2.42	16.53
β-Lactoglobulin A	—	NA	—	NA
β-Casein/α-lactalbumin ratio	12.26	NA	5.77	NA
		Dry milk 100%		
β-Casein	1.55	7.74	10.24	11.56
α-Lactalbumin	0.11	4.62	1.59	11.40
α-Casein 1	1.43	14.23	6.21	9.10
α-Casein 2	0.55	11.29	2.81	21.40
β-Lactoglobulin A	—	NA	—	NA
β-Casein/α-lactalbumin ratio	14.45	NA	6.44	NA

[a] NA, Not applicable.
[b] —, Not detectable.

Experiments showed that phosphate buffer alone cannot resolve caseins by CE technique because of aggregates of caseins. To prevent this aggregation, urea was added to the buffer to effectively separate milk caseins by ion-exchange chromatography (17). Thus, in the presence of 4M urea with phosphate buffer at pH 7.0, α- and β-caseins are well-separated, and α-lactalbumin and β-lactoglobulin A can be identified in fresh nonfat milk, as shown in Figure 3A. β-Lactoglobulin B comigrates with α-casein, but k-casein, which should migrate slightly ahead of β-casein (using pure k-casein), appears to be absent from fresh milk. Figures 3B and 3C show the electropherograms of fresh low-fat and homogenized milks, respectively. Major milk proteins, such as α- and β-casein, α-lactalbumin, and β-lactoglobulins, can be separated in less than 10 min. The distribution of all major protein species in fresh milk, regardless of fat content, appears to be fairly constant. β-Lactoglobulin A migrates at about 7 min at the front of a rather broad peak in low-fat and whole milks (Figures 3B and

3C, respectively). Presumably, this migration zone belongs to minor proteins associated with lipids in milk, similar to those seen in Figures 2B and 2C. For a reconstituted powder milk, the electropherogram in Figure 3D shows the separation of α- and β-caseins, with a small peak of α-lactalbumin in between the caseins that is substantially less than that in fresh milk. β-Lactoglobulin A in the reconstituted milk is also reduced significantly. These results are consistent with the earlier observations that whey proteins in fresh milks are well-resolved with pH 6.0 phosphate buffer, whereas those in reconstituted milks give ill-defined broad peaks, presumably because of process-induced denaturation (Figure 2).

The above experimental data show that all fresh milks contained similar amounts of each protein fraction, and the α-lactalbumin contents were substantially higher than those of dry milk. Separation of proteins in fresh and reconstituted dry milks also indicates that β-casein and α-lactalbumin are well-separated, each with well-defined peaks. Empirically, the ratio of β-casein to α-lactalbumin was constant for all fresh milks. This ratio was 2.6 when based on peak heights and 5.7 when based on peak areas. These particular ratios prove to be a good indicator for determining the amount of dry milk added to the fresh: the higher the ratio, the more powdered milk is present in the sample (Table 1). For example, in our experiment with triplicate runs, the area ratio is 5.7 for pure nonfat milk, 7.7 for 75% nonfat and 25% powder mixture, 10.2 for 50% nonfat and 50% powder mixture, and 12.3 for 75% powder and 25% nonfat mixture. The results may potentially be used to detect the adulteration of fresh milk with powdered milk.

Conclusion

The utility of milk protein determination by CE in an untreated fused-silica column, using a phosphate–urea buffer system, is evident from this report. With this buffer, separation of major milk proteins can be performed rapidly and reliably in an untreated fused-silica capillary. The separation of milk proteins by the CE technique has many advantages over both gel electrophoretic separation and liquid column chromatography. It is convenient and substantially faster than gel electrophoresis (4), and it accomplishes the task of separating both whey proteins and casein proteins, something that liquid chromatography (17) cannot do. Because most protein quantitation and separation techniques are too inconvenient for dairy plants, payments for milk at present are mostly based on the fat content instead of the protein content. By using the CE system, the dairy industry may grade raw milk by its nutritional value based on protein content. Furthermore, whey proteins appear to be fully intact in fresh milk regardless of fat content, whereas these proteins in reconstituted powdered milk are extensively denatured. Determination of whey proteins in milk may potentially be used for monitoring freshness of milk. Using the ratio between β-casein and α-lactalbumin, the adulteration of fresh milk by powdered milk can be detected with a simple automated CE procedure.

Acknowledgments

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Introduction

Pesticide regulatory issues are a national concern. The Annual Florida Pesticide Residue Workshop is dedicated to these concerns and highlights the analysis of pesticide residues and related technology in regard to food and environmental safety. The 3 papers published here are representative of the workshop's most recent proceedings.

The first paper covers estimating dietary exposure from pesticides. When designing food surveys for the purpose of estimating dietary exposure from residues in food as eaten, the effect of the limit of detection (LOD) on exposure assessment should be evaluated before the analytical phase of the food survey is begun. Loftus et al. use computer models to describe exposure as the product of consumption and the magnitude of the residue in food. Examples of controlled field studies are presented to determine optimum LODs.

Improper disposal of pesticides and other toxic chemicals are also concerns of the public and regulatory agencies. In the second paper, Prassad proposes mathematical models to predict the mobility of pesticides belonging to a particular category or family of compounds in the environment. His observations include the characteristic chemical migration of compounds between environmental media.

In the third paper, the results of a pesticide residue screening program reported by Schattenberg and Hsu are typical of U.S. Food and Drug Administration and state regulatory agency findings. A total of 6970 produce samples were screened for 111 pesticides. The results indicate that the food supply in the United States is safe.

PROCEEDINGS OF THE ANNUAL FLORIDA PESTICIDE RESIDUE WORKSHOP

Effect of the Limit of Detection on Exposure Assessment

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An important component in evaluating the risk from pesticides in our foods is estimating the dietary exposure from the pesticide. This paper examines the effect of the limit of detection (LOD) on dietary exposure assessment. When designing food surveys for the purpose of estimating exposure from residues in foods as eaten, the effect of the LOD on exposure assessment should be evaluated before commencement of the analytical phase of the food survey. The purpose of the evaluation is to determine the LOD required to adequately quantify human exposure. The evaluation entails either determining the level at which the LOD is low enough to obtain exposure estimates that fail in the acceptable range or the level at which further reduction of the LOD will not result in additional observable reduction in estimates of exposure. Models describing exposure as the product of consumption and the magnitude of the residue in food are presented. Residue data from controlled field studies are used as examples to determine the optimum LOD to use in a food survey for assessment of exposure from a chronic adverse health effect. A computer program, EXPOSURE 1®, based on the U.S. Department of Agriculture Nationwide Food Consumption Survey, is used to estimate dietary exposure.

Whether or not a pesticide will cause an adverse health effect is dependent on the biochemical activity of the chemical and on the level and duration of exposure to the chemical. No matter what the toxicological properties of the chemical, the level and duration of exposure are critical elements in assessing the potential risk. This paper examines the effect of the limit of detection (LOD) on dietary exposure assessment and, in particular, how to determine the optimum LOD to use in a survey designed to estimate exposure from pesticide residues in foods.

Food surveys are used instead of the estimate provided by controlled (e.g., crop field and processing) studies when a more realistic exposure estimate is needed. Food surveys yield better information on residues in foods as eaten by the consumer than field or processing studies, because the samples are collected in grocery stores or distribution centers versus at the farm gate or processing plant. Therefore, the samples collected in food surveys reflect the effects of commercial handling, washing, and storage on the residue level in the food, and thus, more closely reflect the actual exposure to the consumer.

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Presented at the 28th Annual Pesticide Residue Workshop, St. Petersburg Beach, FL, July 14–17, 1991.

METHOD

Definition of the LOD

When designing the analytical chemistry phase of the food survey, an important question to answer is what residue level should be used for the LOD. The LOD of a method is defined as the lowest concentration that can be determined to be statistically different from a blank. The Guidelines for Exposure Related Measurements proposed by the U.S. Environmental Protection Agency (EPA) (1) follow the American Chemical Society's recommendation (2) of setting the LOD at 3 standard deviation units above the mean value of the blank measurements. The LOD is distinguished from the limit of quantitation (LOQ) in that the LOQ is the level above which quantitative results can be obtained with a specified degree of confidence. The same guidelines recommend setting the LOQ at 10 standard deviation units above the mean value of the blank measurements. In this paper, the effect of the LOD on the exposure assessment is examined, but the same argument also can be used to examine the effect of the LOQ.

Of course, the lower the LOD (or the LOQ) of a method, the closer the measurement of low-level residues is to the true value, and the more accurately the residue profile is defined. However, lowering the LOD of a method comes at a price; even if possible, it can be a difficult and expensive process. The optimum LOD to use in a food survey weighs the difficulty associated with lowering the LOD against the potential effect upon the estimate of exposure used in the risk assessment. The optimum LOD to use in a food survey is greater than or equal to the highest residue level that will result in a significant decline in the exposure estimate. It is dependent on the magnitude and distribution of the residue data in the foods of interest, the level of consumption in the foods of interest, the market share for the use of the chemical on the crops of interest (percentage of crop treated), and the level of exposure associated with acceptable risk.

Residue Data from Food Surveys vs Controlled Crop Field Studies

To assess the optimum LOD, residue data from either food surveys or controlled (e.g., crop field) studies can be used. As

discussed above, samples from food surveys are usually collected in stores or food distribution centers, and thus, are closer to the consumer than samples from controlled field studies collected at the time of harvest. Another key difference between residue data from food surveys and controlled crop field studies is that the treatment history of the samples is usually not known for food survey residue data, whereas the treatment history, including the application rate, number of applications, and time between treatment and harvest are known for controlled field study residue data.

The advantage to using residue data from controlled field studies is that these data are often available because they are required by EPA to set tolerances; whereas food survey results are rarely accurate. The disadvantage to using field study residue data to determine the optimum LOD for a food survey is that, whereas the treated samples from food surveys reflect commercial use patterns selected to confine a pest, the treated samples from controlled studies reflect use patterns designed to establish tolerances (i.e., "worst case" or the theoretical maximum residue concentration). Even though the available data from controlled studies are heavily weighted toward "worst-case" treatment regimens, the resources to conduct a pilot survey often are not available, and residue data from controlled studies must be used. For this reason, we used residue data from a controlled field study to serve as an example of the derivation of the optimum LOD.

Censored Residue Data

Residue data sets, whether generated by food surveys or by controlled field studies, often contain nondetectable residues (i.e., left censored data). More specifically, while a certain proportion of the residues can be detected, all that is known about the remainder of the residues is that they are below the LOD. Thus, to estimate the anticipated residue level, some assumptions must be made about the residue levels in samples with nondetectable residues. In exposure assessment, there are several different ways to treat data reported below the LOD (1, 3–6). The method used will depend on the particular residue data set and on whether market share data are available. Typical methods of dealing with nondetects are (*1*) to assign the value of ½ LOD to all samples with nondetectable levels of residue (typical method when using controlled field data to estimate the residue present in treated foods), (*2*) to assign ½ LOD to a percentage of samples with nondetectable levels and zero to the remaining nondetectable levels (typical method for food survey data), and (*3*) to use statistical models based on the distribution of residues above the LOD. The last method would provide the best estimate of the magnitude of the residues: however, when only a small proportion of the residues are above the LOD, the statistical distribution that best represents the residue data becomes almost impossible to determine. For this reason, one of the first 2 methods is often more convenient to use. The first method is used when all the samples are known to have been treated. The second method is used when the treatment history of the samples is not known; i.e., the samples could have been either treated or not treated. Because untreated samples are not expected to contain residues, a certain proportion of the samples with nondetectable residues are assigned the residue value of zero.

Exposure Equation

Exposure to a chemical in a particular food is the product of the concentration of the chemical in that food and the amount of food consumed. Both the amount of food an individual is likely to consume and the amount of residue found in the food are variable. More specifically, the exposure random variable (E) is defined as the product of the consumption (C) and residue (R) random variables.

$$E = C \times R \tag{1}$$

where C and R are defined as:

$$C = \begin{cases} C_i \text{ with probability } q_i;\ C_i > 0,\ i = 1,\ldots, n \\ 0 \text{ with probability } q_{n+1} \end{cases} \tag{2}$$

where:

$C_i = i^{th}$ level of consumption; $i = 1,\ldots, n$

q_i = probability that an individual would consume amount of C_i of food of interest.

$$R = \begin{cases} r_j \text{ with probability } p_j;\ r_j > 0, \text{LOD},\ j = 1,\ldots, k \\ \text{LOD/2 with probability } [\ 1 - \sum_{j=1}^{k} p_j\ ] \end{cases} \tag{3}$$

where:

r_j = the j^{th} detectable level of residue

p_j = the probability that food of interest contains residues of the chemical of interest in amounts r_j.

A randomly selected individual is either going to consume some amount (C_i) of the food of interest, with the probability or likelihood of this event denoted by q_i, or not consume any amount of that food, and thus not be exposed to the chemical from that particular food, with the probability of this event denoted by q_{n+1}. Furthermore, a food sample in a controlled field study is either going to contain some detectable level (r_j) of the chemical of interest, with the probability of this event denoted by p_j, or contain a nondetectable amount of the residue, with the probability of this event denoted by:

$$[\ 1 - \sum_{j=1}^{k} p_j\ ]$$

When data generated from a controlled field study are to be used to estimate exposure to foods as available to the consumers, the residue random variable defined in Equation 3 is modified to include the effect of the market share or percentage of crop treated of the pesticide on the crop of interest. Let R^* de-

note the random residue variable corrected for the percentage of crop treated, where R^* is defined as:

$$R = \begin{cases} r_j \text{ with probability } P \times p_j;\ r_j > LOD,\ j = 1,\ldots, k \\ LOD/2 \text{ with probability } P \times [\ 1 - \sum_{j=1}^{k} p_j\ ] \\ 0 \text{ with probability } (1 - P) \end{cases} \quad (4)$$

where P = percentage of crop treated.

The probabilities associated with residue levels both below and above the LOD are adjusted for the likelihood that the particular food has been treated with the chemical of interest. Also, the possibility that a food sample is not going to contain any residues is added to the list of possible events, with the probability of this event denoted by (1 – P).

The exposure random variable results from combining each consumption level with each residue level. Assuming that the consumption and residue random variables are independent, i.e., the amount of food consumed by an individual is not related to the amount of residue found in that food, the probability associated with each exposure level is obtained as the product of the probabilities associated with the corresponding consumption and residue levels. Thus, the exposure (E) is defined as:

$$E = \begin{cases} C_i \times r_j \text{ with probability } P \times q_i \times p_j \\ C_i \times LOD/2 \text{ with probability } P \times q_i\ [\ 1 - \sum_{j=1}^{k} p_j\ ] \\ 0 \text{ with probability } v\ [(1 - P) \times (1 - q_{n+1}) + q_{n+1}] \end{cases} \quad (5)$$

where $i = 1, \ldots, n$; $j = 1, \ldots, k$.

Exposure Equation Adjusted for Chronic Effects

A dietary exposure assessment strives to obtain the most accurate estimates of the consumption of the various foods of interest and the magnitude of the residue in those foods. The mathematical models used to compute these estimates in the exposure equation are determined by the nature of the potential risk. The question must be asked whether the adverse health effect results from a long-term exposure (chronic effect) or to one or a few critical exposures (acute effect). The examples in this paper compute exposure for a chemical with chronic effects. Similar principles are applicable for chemicals with acute effects.

To estimate the level of consumption of a food, Technical Assessment Systems, Inc., has created the EXPOSURE Series® (7), a microcomputer version of EPA's Dietary Risk Evaluation System (DRES) (8). The EXPOSURE Series uses data from the U.S. Department of Agriculture's nationwide survey of individual food consumption, conducted every 10 years (9). This survey documents the 3-day dietary records of individuals, as well as socioeconomic and demographic data. EXPOSURE 1 is used to assess chronic exposure. The annualized mean daily consumption for the United States and 22 population subgroups is used as the estimate of consumption of a particular food. The EXPOSURE 1 analysis takes into account that each individual in the population does not necessarily eat a particular food every day and that some people do not eat that food at all. The exposure can be calculated for individual foods and for several foods combined.

The best estimate for the magnitude of the residue from controlled field studies for a chronic exposure assessment usually is the mean residue level corrected for the percentage of crop treated with a particular pesticide. The mean residue level of the pesticide takes into account that an individual consumer of a food would, over many years, be exposed to the average residue level for a particular food treated with the chemical of interest and would be likely to consume the food item both treated and untreated.

Thus, $\bar{E}$, the average of the exposure random variable (E) defined in Equation 5, can be used to represent chronic exposure. $\bar{E}$ is defined as the product of the mean consumption ($\bar{C}$) and the mean residue level corrected for the percentage of crop treated ($\bar{R}^*$).

$$\bar{E} = \bar{C} \times \bar{R}^* \quad (6)$$

where:

$$\bar{C} = \sum_{i=1}^{n} C_i \times q_i \quad (7)$$

and

$$\bar{R} = P \times [\ \sum_{j=1}^{k} r_j \times p_j\ ] + [\ \frac{LOD}{2} \times P \times (\ 1 - \sum_{j=1}^{k} p_j\ )] \quad (8)$$

Substituting Equations 7 and 8 into Equation 6, $\bar{E}$ becomes:

$$\bar{E} = [\ \sum_{i=1}^{n} C_i \times q_i\ ] \times P \times [\ \sum_{j=1}^{n} r_j \times p_j\ ] \quad (9)$$

$$+ [\ \sum_{i=1}^{n} C_i \times q_i\ ] \times \frac{LOD}{2} \times P \times (\ 1 - \sum_{j=1}^{k} p_j\ )\ ]$$

Results

To illustrate how the optimum LOD is derived, we used residue data sets from controlled field studies that investigated the use of a pesticide we will refer to as chemical "X" on apples, bananas, and cucumbers. The assumption is that the proportion of detectable residues among the treated portion of the crops would be the same in samples from the grocery stores as from

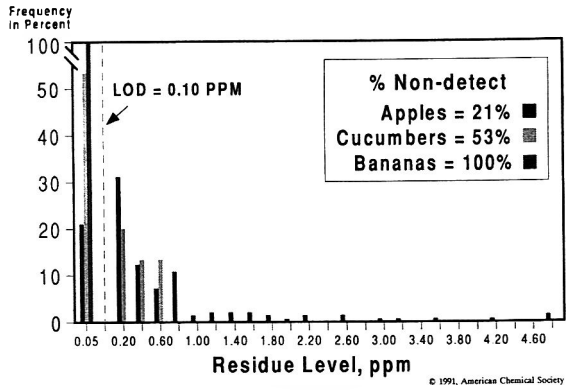

Figure 1. Residue data profile for chemical "X" on apple, bananas, and cucumbers.

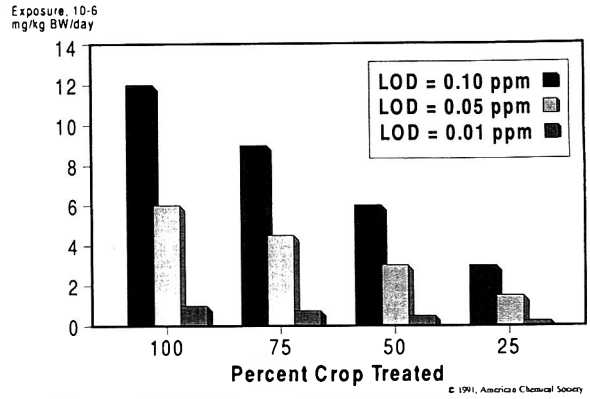

Figure 2. Bananas (nondetectable residues = 100%): Estimate of average exposure to chemical "X" vs LOD and the percentage of crop treated using EXPOSURE 1 (U.S. population).

the controlled field trials. These residue data sets were selected because different residue distributions were observed for chemical "X" on the 3 crops. All the banana samples had nondetectable residue levels, while 53 and 21%, respectively, of the cucumber and apple samples had nondetectable residue levels. The residue profiles of chemical "X" on apples, bananas, and cucumbers (frequency of the observation vs residue level) are shown in Figure 1. Samples with residue levels below the LOD were plotted at the level of ½ LOD.

Using EXPOSURE 1, estimates of the average amount of exposure to chemical "X" were calculated for each commodity by using the mean residue level determined from the residue data depicted in Figure 1. For each commodity, 12 different exposure estimates were calculated for the U.S. population, under 4 different assumptions for the percentage of crop treated (100, 75, 50, and 25%) and 3 different assumptions for the LOD (0.10, 0.05, and 0.01 ppm).

The resulting 12 exposure estimates for chemical "X" on bananas are graphically depicted in Figure 2 for the U.S. population. For bananas, no detectable residues of chemical "X" were found. The proportion of samples not treated were assumed to contain 0 residues, and the remaining samples were assumed to contain residues at ½ LOD. This graph shows that lower values of both the percentage of crop treated and the LOD result in a lower estimate of average exposure. However, the absolute decline in the estimate of exposure associated with a decline in LOD decreases as the percentage of crop treated decreases.

Figure 2 can be used as a guide to select the optimum LOD for chemical "X" on bananas by comparing the exposure estimate in the graph to the exposure level that is associated with acceptable risk. If the acceptable average exposure to chemical "X" on bananas is 5×10^{-6} mg/kg body weight/day and the pesticide has widespread use—approaching 100%—one would need to lower the LOD to some level below 0.05 ppm (namely, 0.04 ppm) to obtain an estimate of average exposure in an acceptable range. On the other hand, if the percentage of crop treated is 50–75%, lowering the LOD to 0.05 ppm results in exposure estimates that are in an acceptable range—even assuming that there are no detectable residues. Finally, if only 25% of the crop is treated, an LOD of 0.1 ppm is adequate to obtain exposure estimates in the acceptable range; there will be no reason to invest time and money to lower the LOD.

Figure 3 contains estimates for chemical "X" on apples under the same scenarios as for bananas in Figure 2. For apples, 21% of the residues were nondetectable. Again, lower estimates of the percentage of crop treated yield lower estimates of average exposure. However, for a given value of the percentage of crop treated, a lower LOD does not result in an *observable* effect on the estimate of average exposure. This is because a finite residue level greater than 0.1 ppm was observed for 80% of the samples, and because much higher exposure values were observed for apples relative to the difference in the exposure estimate at each LOD value. Thus, the resources required to lower the LOD below 0.1 ppm would not significantly affect the exposure estimate. Resources would be better spent to refine estimates of the other components of the risk, such as the intrinsic toxicological activity or the market share of the pesticide. Ultimately, if the most refined risk assessment indicates that the exposure is not at an acceptable level, resources could be expended on studies to determine new use patterns that would reduce the magnitude of the residues but still confine the pest, and, thus reduce the actual exposure.

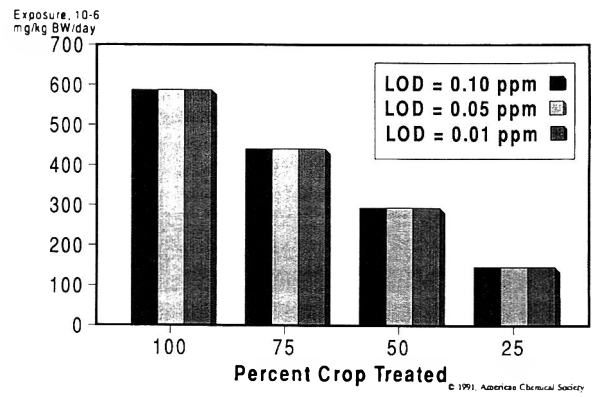

Figure 3. Apples (nondetectable residues = 21%): Estimate of average exposure to chemical "X" vs LOD and the percentage of crop treated using EXPOSURE 1 (U.S. population).

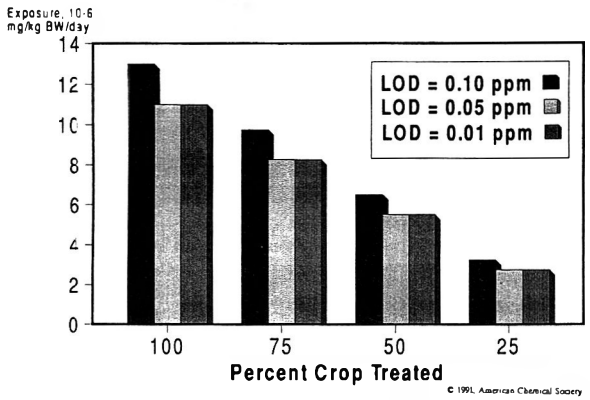

Figure 4. Cucumbers (nondetectable residues = 53%): Estimate of average exposure to chemical "X" vs LOD and the percentage of crop treated using EXPOSURE 1 (U.S. population).

The 12 exposure estimates for chemical "X" on cucumbers are graphically depicted in Figure 4 for the U.S. population. For cucumbers, 53% of the residues were nondetectable. This graph shows that lowering the LOD from 0.10 to 0.05 ppm results in an observable decline in the estimate of average exposure, and that the absolute decline in this estimate decreases as the value of the percentage of crop treated decreases. However, lowering the LOD from 0.05 to 0.01 ppm does not result in an observable decline in the estimate of average exposure because of the relatively large magnitude of the detectable residues and the relatively low levels of consumption of cucumbers on an annualized basis.

If the allowable average exposure to chemical "X" on cucumbers is 12×10^{-6} mg/kg body weight/day and if the pesticide has widespread use approaching 100%, one would need to lower the LOD to 0.05 ppm to obtain an estimate of average exposure in an acceptable range. However, it would not be worthwhile to lower the LOD to 0.01 ppm, because an observable effect on the exposure estimate would not be achieved. If 75% or less of the crop is treated, an LOD of 0.1 ppm is adequate to obtain exposure estimates in the acceptable range; there will be no reason to invest time and money to lower the LOD.

Discussion

In conclusion, the level of detection of the method to be used in a food survey has important implications on study resources, the time requirements, and the utility of the results for assessment of human risk. Because controlled field study residue data to support establishment of tolerances were used to derive the optimum LOD for a food survey, a safety factor was built into this analysis in that higher residue levels are expected from such data, as compared to food survey residue data. If food survey residue data were used to derive the optimum LOD, this safety factor would not be built-in, and, in certain cases, an LOD somewhat lower than that determined in this sort of analysis should be used in the survey. The reason for this is that the residue profile of the data used to derive the optimum LOD is not the actual residue data profile but an estimate of the profile. In a future paper, we will use food survey residue data to derive the optimum LOD.

The examples in this paper are based on a single type of risk, the chronic exposure to a chemical. The process can be applied, with some simple modifications, to acute exposure. Although in this paper we considered only the mean residue level and the mean consumption, the exposure is really a distribution of exposure levels resulting from the combination of the distribution of consumption levels of a food and the distribution of residue levels of that food (as described above in Equation 5). Technical Assessment Systems, Inc., has developed computer programs that use distributions to represent the consumption and the magnitude of the residue. The mechanics of the analysis are more complicated, but the principles are the same. Computer programs, such as the TAS EXPOSURE Series, are available that greatly simplify the process.

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PROCEEDINGS OF THE ANNUAL FLORIDA PESTICIDE RESIDUE WORKSHOP

Predicting the Environmental Distribution of Compounds with Unknown Physicochemical Properties from Known Pesticide Properties

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Disposing of pesticides improperly can have environmental consequences. This study examines those properties discussed in published literature or derived from semiempirical mathematical models that are most likely to affect pesticides' environmental distribution, then assesses their migration between environmental media (soil, water, air, and biota). In particular, it examines how pesticide characteristics such as water solubility, molecular weight, bioconcentration, volatility, and soil adsorption affect soil-to-water mobility, water-to-air dissipation, and water-to-biota accumulation when present in the environmental medium of preferred residence. The study concludes that chemicals that have low water solubilities tend to adsorb to soil, those that have low vapor pressures tend to dissipate slowly from water, and those that have relatively high octanol-to-water partition coefficients or low water solubility have a high potential for bioconcentration. Under these situations, environmental restoration might best be achieved by removal of the contamination at the source. By drawing analogies from these findings, researchers should be able to predict the mobility of pesticides belonging to a particular category or family of compounds with unknown physicochemical properties, determine the harm that might result from their distribution under worst-case scenarios, and recommend ways to restore the environment.

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The occurrence of pesticides in groundwater and, more specifically, in drinking water wells prompted the U.S. Environmental Protection Agency (EPA) to produce a National Pesticide Survey (NPS) (1). Another EPA study, sponsored by the Office of Groundwater Protection, resulted in the development of a guidance document on protecting groundwater from pesticides and agricultural practices (2). It deals with mitigation practices used to alleviate the groundwater contamination that can be caused by the normal use of chemical pesticides in agriculture. Adverse environmental consequences are also possible when pesticides are used or disposed of in a manner inconsistent with the directions given on their labels (a practice mandated by the Federal Insecticide, Fungicide, and Rodenticide Act).

The focus of the study discussed here was to examine some basic pesticide properties that are most likely to influence their environmental distribution, to examine the pesticides' affinities for movement between environmental media (air, water, soil, and biota) and qualitatively assess their mobility in the environment, and to predict the environmental distribution of compounds with unknown physicochemical properties under a worst-case disposal scenario on the basis of properties of other compounds or categories of compounds. Information for this study was derived from published literature as well as from interpretations of estimations made using published semiempirical mathematical models, when necessary.

Hopefully, the findings from this study will provide useful information for overall risk assessments and endangerment characterizations. These studies are conducted to determine the magnitude and probability of actual or potential harm to public health and welfare or the environment that could or does result from releases of hazardous chemicals under scenarios involving uncontrolled waste sites (3). The findings from this study may also help environmental professionals recommend ways to remedy problems and restore the environment, when deemed necessary, by making risk-based comparisons of various alternatives considered for site cleanup.

Concept

After a pesticide finds its way into the environment, it is mainly distributed among the following 3 environmental

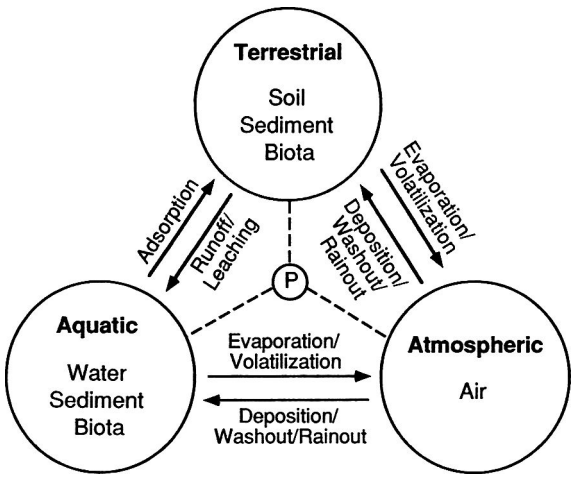


Figure 1. Environmental distribution and intercompartmental transfer of pesticides.

media: air or atmospheric compartment, surface-water or aquatic compartment, and soil or terrestrial compartment (Figure 1). A fourth medium, the biotic compartment, is considered in conjunction with surface water.

The concentration of the pesticide initially present in each compartment is a function of its source, the type of release, and the pesticide's properties. Once present in the environment, the pesticide undergoes changes that are a function of intercompartmental transfers, transformations, and degradations. If the pesticide is mobile in the environment, it may migrate to the air, surface water, or groundwater, where uptake by terrestrial or aquatic organisms is possible. Consequently, through biomagnification, concentrations of the pesticides in biological tissues can reach a level that may cause a significant toxicological hazard to humans and other populations.

METHOD

A partition analysis approach, which has been proposed for use as a tool in managing hazardous waste studies, was used to assess the environmental distribution of chemicals (4). On the basis of the assumption that no degradation occurs, theoretically, the total concentration or equilibrium concentration of the chemical can be the sum of its concentration in each environmental medium to which it is exposed; that is, the sum of the concentration in soil, water, air, biota, and others, as hypothesized, and it may be represented by the following equation:

$$C_T \text{ or } C_E = f(C_{Soil}, C_{Water}, C_{Air}, C_{Biota}, \text{other})$$

Table 1. Environmental monitoring status of selected pesticides

	Analyte on NPS[a]			Reported occurrence[c]		
Pesticide	Primary	Other	CLP and TCL[b]	GW	SW	Soil
Aldicarb	•[d]	•	—[e]	—	—	—
Aldrin	—	•	•	•	—	•
Atrazine	•	•	—	—	—	—
Carbaryl	•	•	—	—	—	—
DDT	—	•	•	•	•	•
DDD	—	•	•	•	•	•
DDE	—	•	•	•	•	•
Disulfoton	•	•	—	—	—	—
Heptachlor	•	•	•	•	—	•
Methoxychlor	•	•	•	—	—	•
Prometon	•	•	—	—	—	—
Prometryn	—	•	—	—	—	—
			Substituted benzenes			
Monochlorobenzene	—	—	—	—	—	—
1,2-Dichlorobenzene (ortho)	—	—	—	—	—	—
1,3-Dichlorobenzene (meta)	—	—	—	—	—	—
1,4-Dichlorobenzene (para)	—	—	—	—	—	—
1,2,4-Trichlorobenzene	—	—	—	—	—	—
1,2,4,5-Tetrachlorobenzene	—	—	—	—	—	—
Hexachlorobenzene	•	•	—	—	—	—

[a] Analyte listed on EPA's March 1990 National Pesticide Survey (NPS) (1). Primary reflects potential occurrence in drinking water wells.
[b] The Contract Laboratory Program (CLP) and Target Compound List (TCL) are based on the Comprehensive Environmental Response Compensation and Liability Act (CERCLA or Superfund) (3).
[c] Adapted from CLP pesticide data bases (5); GW = groundwater and SW = surface water.
[d] Bullet indicates that pesticide is applicable or has been reported to occur.
[e] Dash indicates that pesticide is not applicable or that no occurrence of it has been reported.

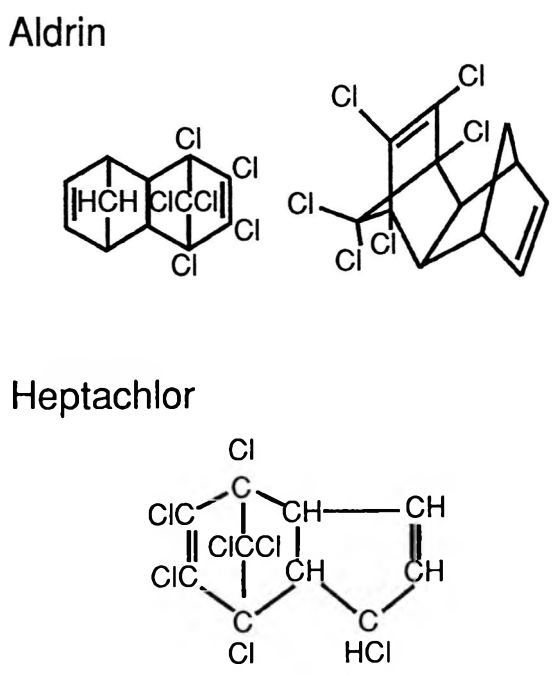


Figure 2. Structure of chlorinated cyclodienes.

where:

C = chemical concentration,
T = total, and
E = equilibrium.

A number of distribution ratios between media can be estimated.

Published partition coefficients or those derived from basic physicochemical properties of chemicals were used to predict the equilibrium distribution in environmental media. The partition coefficients needed included the following:

Between water and soil organic carbon (i.e., soil adsorption coefficient [K_{oc}]),

Between water and air (i.e., Henry's law constant [H]),

Between water and biota (i.e., bioconcentration factor [BCF]), and

Between water and *n*-octanol (i.e., octanol-to-water partition coefficient [log P]).

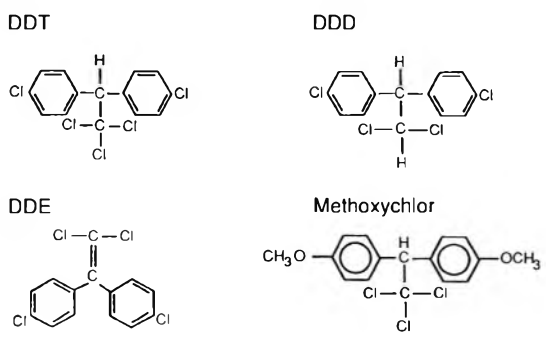


Figure 3. Structure of organochloride compounds.

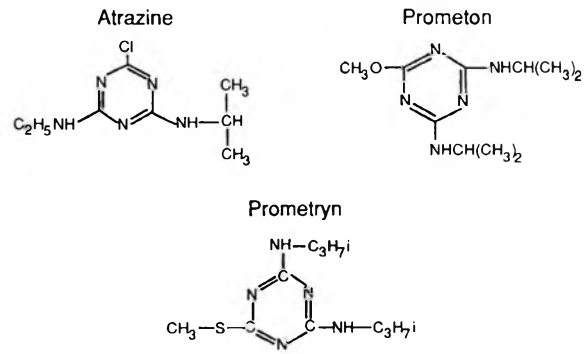


Figure 4. Structure of symmetrical triazines.

Several benchmark chemicals listed in available environmental monitoring data such as the EPA's NPS (1) and the Superfund's Contract Laboratory Program (CLP) data bases (5) were selected for analysis and interpretation (Table 1) (1, 3, 5). All of the chemicals chosen, including those that are of primary importance due to their potential occurrence in drinking-water wells, are among the 127 different analytes listed in the NPS (1). Some of the chemicals are pesticides that are among the 20 different analytes in the CLP target compound list (TCL) and/or pesticides that have been reported to occur in groundwater, surface water, and soil (5). Also included are a category of halogenated hydrocarbons (substituted benzenes) that are prevalent at hazardous waste sites, which serve as an example of a family of compounds. The selected chemicals may be grouped as follows:

Halogenated hydrocarbons comprising chlorinated cyclodienes: aldrin and heptachlor (Figure 2); organochlorine compounds: dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE), 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane (DDD), and methoxychlor (Figure 3);

Symmetrical triazines: atrazine, prometon, and prometryn (Figure 4);

An organophosphate: disulfoton (Figure 5);

Carbamates: aldicarb and carbaryl (Figure 6); and

A family of substituted benzene compounds, in which 1–6 of the hydrogen atoms of benzene is/are replaced by chlorine atoms: monochlorobenzene, 1,2-dichlorobenzene, 1,3-dichlorobenzene, 1,4-dichlorobenzene, 1,2,4-trichlorobenzene, 1,2,4,5-tetrachlorobenzene, and hexachlorobenzene (Figure 7). The selected substituted benzenes are used in the manufacture of organic chemical intermediates, other chlorobenzenes, pesticides, dyes, process solvents, and other materials.

Results and Discussion

Pesticide properties that are of concern because of their influence on environmental mobility include molecular weight, solubility, adsorption, and volatility. Table 2 lists, for selected pesticides, values for molecular weight, water solubility, and vapor pressure (4, 6). Data on these properties are used in fate and transport modeling for environmental contamination assessments or are used to estimate partition coefficients when

Disulfoton

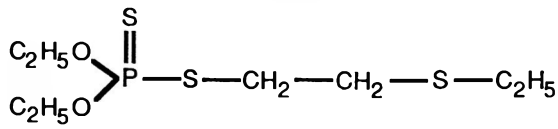


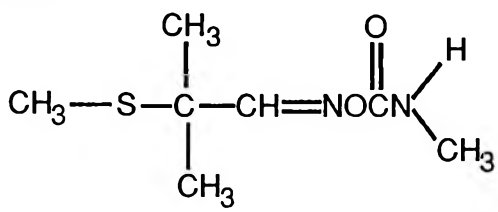
Figure 5. Structure of organophosphates.

experimental data are unavailable. These chemical characteristics can influence a chemical's environmental behavior both directly and indirectly. For example, highly soluble chemicals are likely to be easily and quickly distributed by the hydrologic cycle. Such chemicals tend to have relatively low soil and sediment adsorption coefficients and relatively low BCFs in aquatic life (7).

The vapor pressure of a pure substance provides an indication of its ability to vaporize in an unperturbed environment (8). The vapor pressure of a chemical or its mixture divided by its aqueous solubility provides an estimation of the Henry's law constant (H), which is a useful measure for predicting the exchange rates of vapors across an air-to-water interface. It should be remembered, however, that this relationship is true only when the solubility is small. Values for H are not available for many of the compounds studied because of analytical difficulties associated with measuring this property, and because measurements are often made with only small quantities (nanomoles or less) of the compound.

Most of the data for the parameters listed in Table 2 were adapted from EPA (6); however, data for substituted benzenes were adapted from Prasad and Whang (4). On a relative basis, of the chemicals assessed, aldrin has the largest molecular weight (365) and monochlorobenzene the least (112.56); aldicarb has the highest solubility (60 000 mg/L) and DDD and DDT the least (5.0×10^{-6} and 1.28×10^{-3} mg/L, respectively). Aldicarb has the lowest H (1×10^{-10} atm^3/mole) and monochlorobenzene the highest (1×10^{-1}). These data were used either directly for assessment or indirectly for estimating partition coefficients when published data were unavailable. Table 3 gives measures of soil adsorption, soil-to-water mobility, and relative soil volatility, as well as a waste categorization based on soil volatility data (4, 6, 9).

Aldicarb



Carbaryl

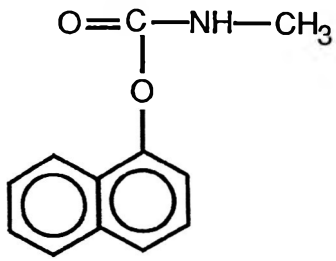


Figure 6. Structure of carbamates.

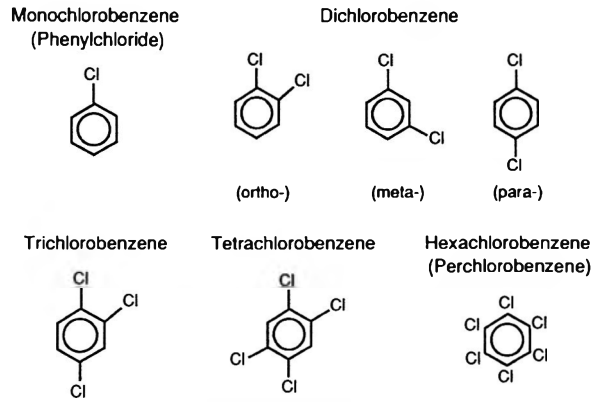


Figure 7. Structure of substituted benzenes.

Soil-to-Water Partitioning

Each chemical's intercompartmental partitioning between soil (organic carbon) and water was assessed based on its soil adsorption characteristic, expressed as K_{oc} (Table 3). Soil adsorption as discussed here is the phenomenon by which a liquid or gaseous material concentrates on the surface of a soil particle (in particular, the organic matter). As such, K_{oc} values are soil dependent for a particular chemical. The reverse process (the removal or movement of ions or gaseous or liquid material from the solid phase into soil solution) is referred to as desorption.

The soil adsorption coefficient, K_{oc}, is normally determined by classical experiments in which a known amount of chemical is equilibrated with a known amount of soil. The chemical is then extracted from the soil with a known amount of extracting solution. The amount of chemical removed by the solution allows calculation of the fraction adsorbed by soil. The soil organic carbon is factored into the calculation. These experiments can be verified by fitting the results to an adsorption equation or an adsorption isotherm. For this study, these data were taken from Kenaga and Goring (10), who reported the K_{oc} values for a variety of pesticides as either calculated or experimental. Data for substituted benzenes were taken from Prasad and Whang (4), except for the experimental K_{oc} value for hexachlorobenzene, which was derived from Kenaga (9). Experimental K_{oc} values are likely to differ from calculated K_{oc} values, because the K_{oc} value for chemicals appears to be related not only to soil organic matter but also to soil mineral properties. Soil-to-water mobility of a given compound (e.g., by leaching) is a function of soil adsorption. Kenaga's mobility rating scheme (9) proposes that if a chemical's K_{oc} value is

Table 2. Physicochemical properties of selected pesticides[a]

Pesticide	Molecular weight	Water solubility at ≥20°C (mg/L)	Vapor pressure at 25°C (mm Hg)	Henry's constant (atm^3/mol)
Aldicarb	190	60000	1×10^{-4}	1×10^{-10}
Aldrin	365	0.2	6×10^{-5}	4.96×10^{-4}
Atrazine	215.7	33	3×10^{-7}	—[b]
Carbaryl	201	40	<0.005	1.82×10^{-2}
DDT	354	1.28×10^{-3}	1.5×10^{-7}	5.2×10^{-5}
DDE	—	1.3×10^{-3}	—	—
DDD	320	5×10^{-6}	1.5×10^{-7}	1.26×10^{-2}
Disulfoton	274	25	1.8×10^{-4}	2.59×10^{-6}
Heptachlor	313	0.56	3×10^{-4}	1.48×10^{-3}
Methoxychlor	345.5	0.04	—	—
Prometon	225	750	2.3×10^{-6}	—
Prometryn	241	48	1.0×10^{-6}	—
		Substituted benzenes		
Monochlorobenzene	112.56	500	0.80	1.00×10^{-1}
1,2-Dichlorobenzene (ortho)	147.01	100	1.00	8.05×10^{-2}
1,3-Dichlorobenzene (meta)	147.01	123	1.00	1.24×10^{-1}
1,4-Dichlorobenzene (para)	147.01	79	0.60	4.11×10^{-2}
1,2,4-Trichlorobenzene	181.45	30	0.29	9.60×10^{-2}
1,2,4,5-Tetrachlorobenzene	215.90	4	0.05	9.54×10^{-2}
Hexachlorobenzene	285	0.035	1.25×10^{-5}	1.7×10^{-3}

[a] All data were derived from Ref. 6, except the data for substituted benzenes, which were adapted from Ref. 4.
[b] Dash indicates that data are not available.

≥1000, it is immobile, and if its value is <100, it is moderately to highly mobile. Of the chemicals examined, aldicarb has a low calculated K_{oc} of 32 and a relatively high mobility when compared with DDT, which has an experimental K_{oc} of 238 000, suggesting that it is relatively immobile. All other compounds studied are either moderately mobile, with K_{oc} values at or near 600, or immobile, with K_{oc} values greater than 1000.

The relative soil volatilities for chemicals categorized under the Resource Conservation and Recovery Act (RCRA), as adapted from EPA (6) for all compounds studied, are also presented in Table 3. These data suggest that DDT has a low soil volatility (8.0×10^{-9}) and that monochlorobenzene has a high soil volatility (11.1×10^{-1}) (6); thus, DDT is categorized a nonvolatile waste and monochlorobenzene as a highly volatile waste. All other compounds studied are either slightly to moderately volatile. The data suggest that substances that are categorized as highly to slightly volatile, such as monochlorobenzene and aldicarb, are also moderately to highly mobile when moving from a soil to water medium. These substances, upon disposal at the soil surface, are likely to move rapidly downward into the subsurface if soil conditions are conducive for leaching.

The low K_{oc} value of 30 for aldicarb indicates that this compound's ability to bind onto soil or sediment particles is low. Therefore, it may be transported in solution by water percolating through soil containing the compound. However, if the subsurface environment is impermeable and nonconducive to leaching, the compound may volatilize from the soil surface. When substances having K_{oc} values >1000 exist at the soil-to-water interface, these compounds favor soil over water. Such compounds include organochlorine compounds, heptachlor, disulfoton, and "heavier" chlorinated benzene compounds, including 1,2,4,5-tetrachlorobenzene and hexachlorobenzene. For all other substances, soil-to-water mobility and relative soil volatility may not directly correlate, because the waste categorization in Table 3 was arbitrary.

In general, under the uncontrolled conditions that exist in hazardous waste sites, chemicals that tend to have low water solubilities and high soil adsorption characteristics have low or minimal mobility in a soil-to-water matrix and tend to resist leaching and stay within the terrestrial compartment or soil matrix. However, under this same scenario, pesticides that possess low K_{oc} values tend to leach from soils and can migrate to groundwater. The proposed mobility rating supports this understanding; for example, the organochlorine and organophosphate pesticides are rated as immobile in the soil-to-water phase (9). Although methoxychlor and disulfoton are immobile based on their soil adsorption characteristics, these compounds are still listed in EPA's NPS (1). Symmetrical triazines, chlorinated cyclodienes, and chlorinated benzenes seem to be moderately mobile to immobile in soil, although atrazine, prometon, heptachlor, and hexachlorobenzene are identified as being of primary importance in EPA's NPS (1).

On the basis of their adsorption and soil-to-water mobility characteristics, some generalizations can be made about families of pesticide products (Figure 8). The carbamates studied,

Table 3. Adsorption, mobility, and relative volatility of selected pesticides

Pesticide	Soil adsorption coefficient[a]	Relative mobility (soil to water)[b]	Relative soil volatility[c]	Volatility category (as waste)[d]
Aldicarb	32(C)	HM	7.3×10^{-6}	SVW
Aldrin	410(E)	MM	3.1×10^{-7}	NVW
Atrazine	149(E)	MM	—[e]	—
Carbaryl	230(E)	MM	—	—
DDT	238000(E)	IM	8.0×10^{-9}	NVW
DDD	80500(C)	IM	8.4×10^{-9}	NVW
DDE	55000(C)	IM	—	—
Disulfoton	1780(E)	IM	1.1×10^{-5}	SVW
Heptachlor	30000(C)	IM	1.6×10^{-5}	SVW
Methoxychlor	80000(E)	IM	—	—
Prometon	350(E)	MM	—	—
Prometryn	810(E)	MM	—	—
		Substituted benzenes[f]		
Monochlorobenzene	145(C)	MM	11.1×10^{-1}	HVW
1,2-Dichlorobenzene (ortho)	347(C)	MM	1.2×10^{-1}	MVW
1,3-Dichlorobenzene (meta)	309(C)	MM	1.7×10^{-1}	MVW
1,4-Dichlorobenzene (para)	395(C)	MM	5.5×10^{-2}	MVW
1,2,4-Trichlorobenzene	672(C)	IM	—	—
1,2,4,5-Tetrachlorobenzene	1629(C)	IM	1.5×10^{-4}	SVW
Hexachlorobenzene	3914(E)	IM	6.5×10^{-7}	NVW

[a] Source is Ref. 9, except for substituted benzenes; C = calculated and E = experimental.
[b] Based on the mobility rating method proposed by Kenaga (9); HM = highly mobile, MM = moderately mobile, and IM = immobile.
[c] Source is Ref. 6.
[d] Source is Ref. 6, which categorizes Resource Conservation and Recovery Act (RCRA) wastes according to relative soil volatility (RSV); HVW = highly volatile waste (RSV >1), MVW = moderately volatile waste (RSV $1–10^{-3}$); SVW = slightly volatile waste (RSV $10^{-3}–10^{-6}$), and NVW = nonvolatile waste (RSV $<10^{-6}$).
[e] Dash indicates that data are not available.
[f] Adapted from Ref. 4.

aldicarb and carbaryl, seem to have high to moderate soil mobility. Both of these compounds have been targeted as compounds of primary importance in the NPS because of their occurrence in groundwater or drinking water supplies.

When all families of pesticides studied are examined on the basis of their soil adsorption characteristic, carbamates are found to be mobile, and organophosphates and organochlorines are found to be immobile. Although disulfoton is flagged as an analyte of primary importance, it is not listed on the TCL. Neither are aldicarb, carbaryl, atrazine, prometon, and hexachlorobenzene. In other words, their presence in groundwater may not be directly related to their partitioning property. Other factors, such as site conditions, vulnerability of ground water due to shallow depth, chemical use pattern, and possibly other parameters, are likely to influence their occurrence in groundwater.

If a pesticide is highly volatile and not very soluble in water, it is likely to be lost to the atmosphere, and less of it will be available for leaching to groundwater. Conversely, highly volatile compounds may become groundwater contaminants. However, if they are highly soluble in water and last longer before they are broken down, they are subject to the forces of leaching for a longer amount of time. Many chlorinated hydrocarbons are highly persistent in soil, but they have not been found in groundwater because of their low solubility in water and strong adsorption to soil.

Water-to-Air Partitioning

Water-to-air dissipation rates of selected pesticides were estimated from data on the evaporation half-life of each chemical, which had been either estimated or compiled from literature. Half-life, $t_{1/2}$, is referred to as the time required for dissipation of 50% of the compound from water via volatilization. Estimations were made on the basis of a theoretical model proposed by Dilling (11). The model is useful in predicting the approximate evaporation half-life ($t_{1/2}$) of a chemical in a water body of a given depth of water column. It is applicable to organic compounds with low molecular weights or slight to low water solubilities. Because many of the compounds under study are organic with slight to low water solubilities, the use of this model appears valid. Data for substituted benzenes were adapted from Prasad and Whang (4).

Figure 9 shows relative water-to-air dissipation rate, as determined from the estimated $t_{1/2}$, for each pesticide as well as for pesticide groups. Of the pesticide groups assessed, substituted benzenes appear to have relatively low evaporation half-

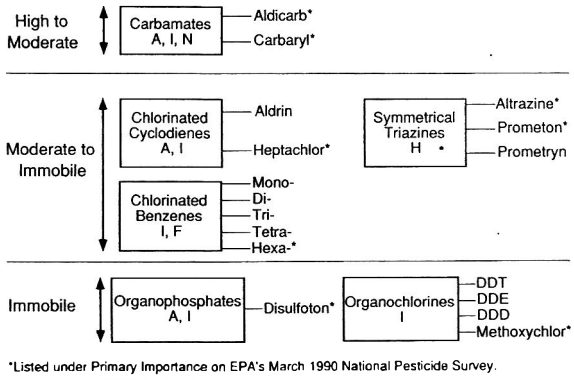


Figure 8. Pesticide mobility in relation to soil adsorption.

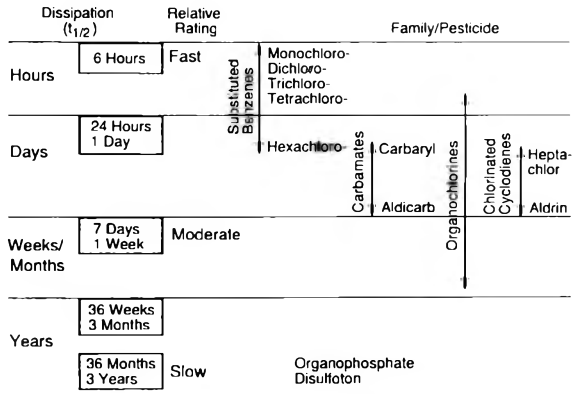


Figure 9. Water-to-air dissipation of selected pesticides.

lives, ranging from about 6 h to slightly more than 24 h. The organophosphate disulfoton appears to have a low dissipation rate, with an approximately calculated $t_{1/2}$ to 36 months. Organochlorines examined appear to show a wide range of $t_{1/2}$, varying from about 12 h to nearly 3 months. This wide variation may be the result of slower photolytic conversions of these products. Carbamates and chlorinated cyclodienes appear to have relatively short ranges of dissipation, ranging from about 1 to 7 days. The exact $t_{1/2}$ for each compound within a pesticide group varies and is possibly dictated by specific physicochemical properties, including molecular weight, water solubility, and vapor pressure.

The above findings suggest that at the water-to-air interface, pesticides with short half-lives, such as lower chlorinated benzene compounds (compounds with less chlorine-substituted benzenes), tend to favor the atmospheric compartment, where they are likely to undergo photodegradations or other conversions or transformations. Those compounds with long half-lives, such as disulfoton, tend to favor or stay in the aquatic compartment and undergo aquatic transformations. However, under field conditions, transformations are dictated by a number of environmental and meteorological factors that should be taken into consideration under real-time situations.

Water-to-Biota Partitioning

Bioconcentration (or bioaccumulation) data were studied to ascertain the relative distribution of selected chemicals found in fish in terrestrial, static-water ecosystems and in trout in flowing-water ecosystems. Bioconcentration is the process in and by which chemicals accumulate in living organisms (8). The ratio of the chemical concentration in a test animal to the concentration in the test environment (water) at steady state is referred to as the bioconcentration factor (BCF). Table 4 gives the published bioconcentration factors for the pesticides discussed here (8, 10, 12, 13). When BCF data were not available, BCF values were estimated using either water solubility or octanol–water partition coefficient data for the chemical. Relative fitting of these properties into regression equation(s) proposed by Kenaga and Goring (10) resulted in BCF values that are useful for interpreting the relative distribution of chemicals in ecosystems.

In both the static-water and flowing-water ecosystems, all chemicals studied exhibit some potential for bioconcentration. Of the chemicals studied, BCF values in flowing-water biota seemed lowest (0) for atrazine and highest (61 000) for DDT. In static-water biota, BCF values seemed lowest (<1) for carbaryl and highest (84 500) for DDT. BCF values are influenced by water solubility, octanol–water partition properties, and the estimation method(s) used.

The BCF data were used to assess the relative distribution of pesticide groups in ecosystems. Figure 10 shows the relative distribution of selected pesticides and pesticide families in static-water ecosystems. The figure allows one to make a quick visual assessment and relative comparison of BCF data in static-water ecosystems. The organochlorines DDT, DDD, and DDE and hexachlorobenzene show a relatively high bioconcentration potential, while the symmetrical triazines, carbamates, organophosphate, and monochlorobenzene show a relatively lower potential for bioconcentration. Chlorinated cyclodienes, the organochlorine compound methoxychlor, and the substituted benzenes (except hexachlorobenzene) exhibit somewhat intermediate bioconcentration potentials in static-water ecosystems when compared with the other pesticide groups.

Figure 11 shows the relative distribution of selected pesticides and pesticide families in flowing-water ecosystems. The trend is somewhat similar to that of static-water ecosystems, with some exceptions. The organochlorines, particularly DDE and DDD, exhibit an intermediate bioconcentration range, with BCF values of about 1614 to 2261. The chlorinated cyclodienes, aldrin and heptachlor, show relatively high bioconcentrations in flowing-water ecosystems, although their BCF values may be somewhat high and result from the estimation method.

Conclusion and Summary

The results discussed above can be used to provide some guidance for managing the environment at disposal sites through the development of migration scenarios for pesticides. The findings, which await further confirmation, may prove useful in allowing predictions to be made of the environmental

Table 4. Bioconcentration of selected pesticides[a]

Pesticide	Input parameter[b]	Bioconcentration factor	
		Flowing water, fish	Static water, trout
Aldicarb	WS, 60000 mg/L	32	41
Aldrin	Log P, 5.17	10800	3140
Atrazine	Log P, 2.67	0	11
Carbaryl	Log P, 2.36	25	<1
DDT	Log P, 6.19	61600	84500
DDD	Log P, 5.96	2261	63830
DDE	Log P, 5.69	1614	27400
Disulfoton	WS, 25 mg/L	26	4
Heptachlor	Log P, 4.40	17400	2150
Methoxychlor	Lop P, 4.67	185	1550
Prometon	WS, 750 mg/L	29	5
Prometryn	WS, 48 mg/L	28	5
	Substituted benzenes		
Monochlorobenzene	Log P, 2.84	12	340
1,2-Dichlorobenzene (ortho)	Log P, 3.38	90	750
1,3-Dichlorobenzene (meta)	Log P, 3.38	90	750
1,4-Dichlorobenzene (para)	Log P, 3.39	91	760
1,2,4-Trichlorobenzene	Log P, 4.17	491	2379
1,2,4,5-Tetrachlorcbenzene	Log P, 4.67	4500	4943
Hexachlorobenzene	Log P, 6.18	8600	44957

[a] Information was derived from the Refs. 8, 10, 12, and 13.
[b] When necessary, BCF values were calculated using regression equation(s) with water solubility (WS) or log P data (10).

mobility of exotic compounds of unknown descent and/or physicochemical properties, by enabling researchers to draw analogies from similar pesticide compounds in the same group or family. This knowledge may help the environmental manager target the environmental media that are most likely to need characterization, cleanup, and restoration.

Pesticides, like other chemical substances, undergo preferential distribution in the environment (Figure 12). This preferential behavior in the environment allows researchers to define major chemical categories. Generally, chemicals that possess large molecular weights, low water solubility, and relatively high octanol-to-water partition coefficients (for example, organochlorine compounds such as DDT [Figure 3]) tend to strongly adsorb to soil and sediment particles and dissipate slowly from water or air and bioaccumulate. Such chemicals become immobile within soil and water, and they also have a relatively high potential for bioconcentration. Environmental releases of chemicals in this category tend to stay near the point of release (i.e., source) or within the medium of release. In soil-to-water systems, effective restoration can be carried out by

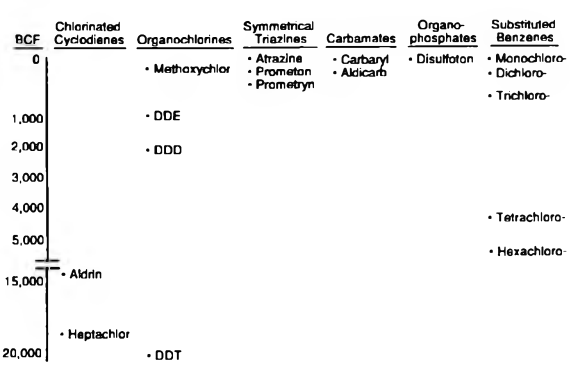


Figure 10. Comparison of pesticide BCFs in static-water ecosystems.

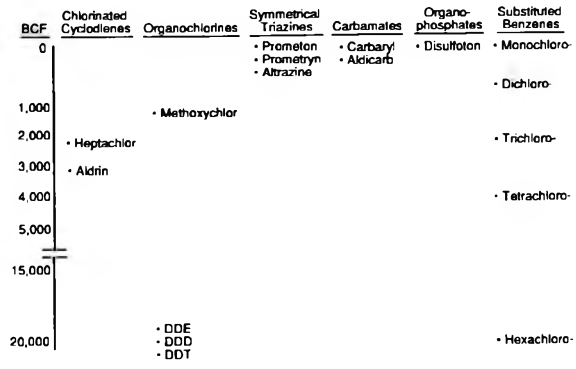


Figure 11. Comparison of pesticide BCFs in flowing-water ecosystems.

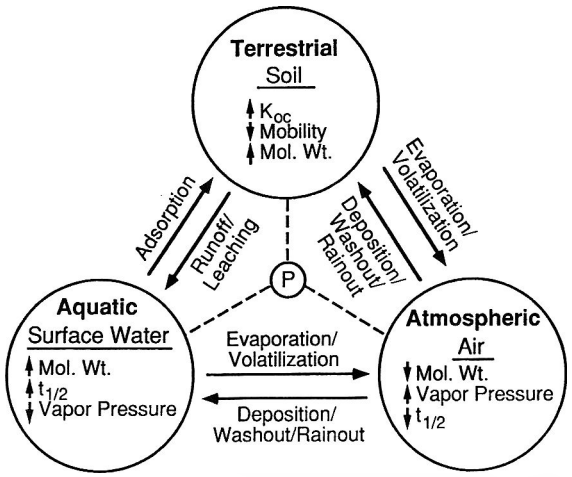


Figure 12. Environmental distribution of pesticides in relationship to physical properties determined by molecular structure.

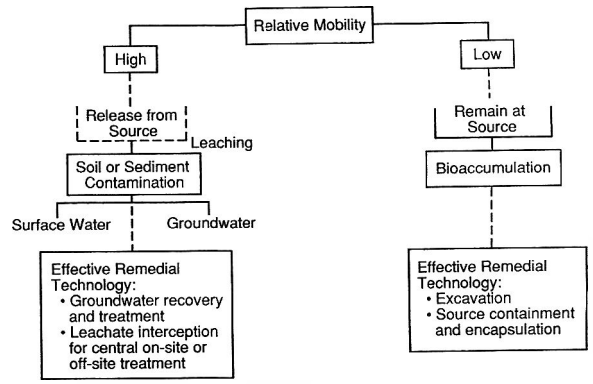


Figure 13. Environmental restoration based on relative contaminant mobility.

excavating the chemical substances or the media contaminated by them, containing the source, or using other remedial technologies, as appropriate (Figure 13).

Conversely, chemicals that possess small molecular weights, are relatively water soluble, and possess relatively low octanol-to-water partition coefficients (for example, monochlorobenzene [Figure 7]) tend to have low soil and sediment adsorption and dissipate relatively quickly from water or air. These properties tend to make the chemicals mobile from soil to water and from water to air, but they also contribute to their relatively low bioconcentration. Environmental releases of chemicals in this category tend to migrate from their source and environmental medium of residence. When these compounds migrate into surface water or groundwater, they may be effectively recovered (for example, by groundwater pumping or leachate interception and treatment). For volatile substances, which favor air pathways in the environment, removal by air or steam stripping or other acceptable methods may be most appropriate.

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Pesticide Residue Survey of Produce from 1989 to 1991

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A pesticide residue screening program for 111 pesticides was performed on 6970 produce samples. Of the 81 varieties of samples, 2.4% contained illegal levels of pesticide residues (that is, higher than U.S. Environmental Protection Agency [EPA] tolerance or no tolerance specified), and 13.3% contained levels within tolerable limits established by EPA. Pesticide results are presented both by commodity and category type. The nature of violative residues is discussed.

Pesticide use in agriculture over the last several decades has proven to be a great benefit to the production of our nation's food supply. Pesticide use has improved both the efficiency of growing crops and the quality of food produced. This has, in turn, lowered the cost of the household food budget. However, along with the benefits emerged the potential effect of trace amounts of pesticide residues remaining on some commodities at the time of sale to the general public. There has recently been concern by consumer groups demanding assurance from the agricultural community that the food we eat is indeed safe. As a response to this concern and for the safety and confidence of its customers, the H.E.B. Food Stores of San Antonio, TX, initiated a pesticide residue screening program in May 1989. The program's purpose was to develop a statistical data base of growers and brokers from which to purchase the produce to supply their stores. Produce that is found to contain illegal levels of one or more residues is rejected from the warehouse and not allowed to proceed to retail stores.

The list of pesticides along with detection limits used on the program are given in Table 1. An illegal residue is defined as one that is either above U.S. Environmental Protection Agency (EPA) tolerance (1) or one that falls above the detection limit listed in Table 1 even though no tolerance is given. This list encompasses the 3 major classes of pesticides: organochlorine, organophosphorus, and *N*-methyl carbamates. In addition, specific residue analyses for EBDCs and dinoseb have been performed on selected commodities. The methodology, along with detection limits used to screen samples, is based upon the California Department of Food and Agriculture (CDFA) Multiresidue Pesticide Screen (2).

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Table 2 lists the wide range of commodity types used in this ongoing study. The categories include, among others, 1205 root and tuber, 646 cucurbits, 625 brassica, 426 bulb vegetables, 419 fruiting vegetables, and 408 pome fruits. The daily samples are picked randomly; however, higher use or seasonal commodities may be more frequently sampled because of their increased frequency of delivery.

METHOD

The modified CDFA procedure, described previously (3), involves an acetonitrile partition extraction followed by exchange to the appropriate solvent for each analyte class. Gas chromatography/electron capture detection was used for organochlorine pesticides, and gas chromatography/nitrogen-phosphorous flame photometric detection was used for organophosphorus pesticides. Carbamate pesticides were determined by using liquid chromatography followed by postcolumn derivatization and fluorescence detection.

Results and Discussion

A total of 6970 samples (approximately 80% domestic, 20% foreign) was taken from the 81 varieties given in Table 2. The philosophy of choosing commodities for daily analysis was random. However, because some commodities are marketed in a much larger volume, many more samples of these were tested, even with random sampling. Again, the purpose of this program is not to sample equivalent amounts of each commodity, but rather to randomly sample what is actually being marketed to the public. Table 3 lists the overall results for the most frequently tested individual commodities. These results are broken down into 2 areas. First, the center 2 columns list the number of residues found within tolerable limits, followed by the right 2 columns, which list the number of residues with concentrations that prohibit use of the commodity (i.e., violations). Table 4 consists of additional information that includes actual residue names and their frequency found on individual commodities both below and above tolerance. The data in these 2 tables indicate that some commodities generally contain a higher percentage of residues when compared to others. This is better illustrated in Table 5, which ranks the 10 commodities with the most frequent rate of violation. Three of the top 5 violating commodities grow in the ground, and 5 of the other 7

Table 1. List of analytes

Compound name (common)	Detection limit, ppm	Compound name (common)	Detection limit (ppm)	Compound name (common)	Detection limit (ppm)
		Organochlorines			
Alachlor (Lasso)	0.5	2,4′-DDT	0.050	Metolachlor	0.020
Aldrin	0.05	4,4′-DDT	0.050	Metribuzin	0.050
Atrazine	1.25	Dichlobenil (Casoron)	0.050	Nitrofen (TOK)	0.100
Bayleton	0.75	Dichlone	0.500	Ozadiazon (Ronstar)	0.100
Benefin (Balan)	0.125	Dichloran (Botran)	0.050	Oxyfluorfen (Goal)	0.100
BHC (Benzahex)	0.05	Diclofop-Methyl	0.100	PCNB	0.040
Bifenox (Modown)	0.25	Dicofol (Kelthane)	0.500	Permethrin	0.050
Bromoxynil	0.5	Dieldrin	0.020	Perthane (Ethylan)	5.000
Captafol	0.25	Dyrene	0.500	Profluralin (Tolban)	0.050
Captan	0.5	Endosulfan I, II, III	0.100	Pronamide (Kerb)	0.040
Chlordane	0.625	Endrin	0.050	Propachlor	0.5
Chlorobenzilate	5.0	Etridiazole	0.100	Propazine	0.250
Chloroneb	0.25	Fluchloralin (Basalin)	0.050	Sonalan (Ethalfluralin)	0.050
Chlorthalonil (Bravo)	0.25	Folpet	1.000	Terbuthylazine	0.500
Cypermethrin	0.125	Heptachlor	0.010	Tetradifon (Tedion)	0.200
Dacthal	0.125	Heptachlor Epoxide	0.020	Toxaphene (Attac)	2.000
2,4′-DDD	0.125	Iprodione (Rovral)	2.000	Trifluralin (Treflan)	0.050
4,4′-DDD	0.125	Lindane (Gamma-BHC)	0.020	Vegadex	0.100
2,4′-DDE	0.075	Linuron	0.200	Vinclozolin (Ronilan)	0.100
4,4′-DDE	0.075	Methoxychlor	0.500		
		Organophosphates			
Acephate	1.25	Dioxathion (Delnav)	0.200	Mevinphos (Phosdrin)	0.100
Azinphos-methyl (Glution)	5.0	Disulfoton (Disyston)	0.100	Monitor	0.200
Bolstar	0.75	EPN	0.300	Naled (Dibrom)	0.200
Carbophenothion (Trithion)	0.5	Ethion	0.100	Nemacur (Phenamiphos)	0.300
Chlorfenvinphos (Supona)	0.75	Ethoprop (Mocap)	0.050	Parathion	0.100
Chlorpyrifos (Dursban)	0.25	Fenitrothion (Sumithion)	0.100	Parathion, Methyl	0.100
Ciodrin	2.5	Fenthion (Baytex)	0.200	Phorate (Thimet)	0.100
Coumaphos (Co-Ral)	2.5	Fensulfothion (Dasanit)	0.250	Phosalone (Zolone)	2.000
DEF (Butifos)	0.25	Fonofos (Dyfonate)	0.100	Phosphamidon (Dimecron)	0.500
Demeton (Systox)	0.25	Hostathion (Triazophos)	0.300	Profenofos (Curacron)	1.000
Dialifor (Torak)	2.5	Imidan	1.000	Propetamphos (Safrotin)	0.100
Diazinon	0.25	Isofenphos (Oftanol)	0.200	Ronnel	0.100
Dichlorvos (DDVP)	0.25	Malathion	0.100	Tetrachlorvinphos	2.000
Dicrotophos (Bidrin)	0.25	Methidathion (Supracide)	0.200	Thionazin (Zinophos)	0.100
Dimethoate (Cygon)	0.25				
		Carbamates			
Aldicarb (Temik)	0.200	Carbaryl (Sevin)	0.200	Methomyl (Lannate)	0.200
Aldicarb Sulfone	0.200	Carbofuran (Furadan)	0.400	Propoxur (Baygon)	0.500
Aldicarb Sulfoxide	0.200	Methicarb (Mesurol)	0.500		

grow on the ground. Also, inspection of Table 4 reveals that a great portion of these positive residues are compounds, such as dieldrin, 4,4′-DDE, and heptachlor epoxide. These materials are no longer applied to crops and are known to have extremely long half-lives in the environment.

The overall violation rate for the 6970 samples taken during this study was 2.4%. This frequency is consistent with or slightly lower than that reported by Yess et al. (4). It is, however, higher than previous reports of 0.71% by CDFA (5) and other CDFA values reported (6). The higher incidence rate of

Table 2. Commodities tested

Apples	Cantaloupes	Endive	Mangos	Persimmons	Sprouts
Apricots	Carrots	Escarole	Melons	Pineapples	Squash
Artichokes	Cauliflower	Garlic	Nopalitos	Plums	Star fruit
Asparagus	Celery	Ginger	Okra	Pomegranates	Strawberries
Avocados	Chard	Grapefruit	Onions	Potatoes	Tangelos
Bananas	Cheriomas	Grapes	Oranges	Prickly pears	Tangerines
Bean sprouts	Cherries	Greens	Papayas	Pumpkins	Tomatoes
Beans	Cilantro	Jicama	Parsley	Quince	Turnips
Beets	Coconuts	Kiwi	Parsnips	Radishes	Watercress
Blueberries	Corn	Kohlrabi	Passion fruit	Raspberries	Watermelon
Bok choy	Cranberries	Leeks	Peaches	Rhubarb	Yams
Broccoli	Cucumbers	Lemons	Pears	Rutabagas	
Brussels sprouts	Diaxone	Lettuce	Peas	Soybeans	
Cabbage	Eggplant	Limes	Peppers	Spinach	

violation can be attributed to several factors. Most important is that this program has adhered strictly to 40 CFR tolerances. Many of the violative residues, such as DDE and dieldrin, would not have been violative if the U.S. Food and Drug Administration guidelines were followed (7).

Table 6 illustrates the 10 commodities with the most frequent rate of detectable residues below EPA tolerance. In this case, the tree bearing fruits (i.e., peaches, plums, nectarines, oranges, and lemons) predominate, leaving carrots as the only root commodity. Carrots are on this list because so many con-

Table 3. Pesticide results by commodity

Commodity	Total samples	Compounds below tolerance	Percent	Violations	Percent
Apples	335	28	8.4	2	0.6
Broccoli	203	17	8.4	5	2.5
Cabbage	295	14	4.7	2	0.7
Carrots	226	82	36.3	13	5.8
Celery	200	69	34.5	1	0.5
Cucumbers	147	31	21.1	16	10.9
Grapefruit	106	32	30.2	0	0.0
Grapes	198	88	44.4	1	0.5
Greens	153	46	30.1	13	8.5
Lemons	139	47	33.8	1	0.7
Lettuce	318	57	17.9	7	2.2
Limes	78	7	9.0	1	1.3
Melons	88	10	11.3	0	0.0
Mushrooms	394	9	2.3	0	0.0
Nectarines	45	18	40	0	0.0
Onions	429	18	4.2	9	2.1
Oranges	220	82	37.3	4	1.8
Peaches	84	35	41.7	1	1.2
Pears	71	7	9.9	1	1.4
Peppers	179	57	31.8	1	0.6
Plums	53	22	41.5	1	1.9
Potatoes	741	55	7.4	14	1.9
Radishes	79	1	1.3	5	6.3
Spinach	112	31	27.7	3	2.7
Squash	290	21	7.2	9	3.1
Strawberries	76	43	56.6	2	2.6
Tomatoes	207	28	13.5	6	2.9
Turnips	44	7	15.9	3	6.8

Table 4. Compounds found on individual commodities

Commodity and (No. of samples)	Compounds below tolerance	No. found	Percent	Compounds with violations	No. F.S.	Percent
Apples (335)	Captan	2	0.6	Carbaryl	2	0.6
	Endosulfan	14	4.2			
	Ethion	11	3.3			
	Parathion	1	0.3			
Broccoli (203)	Carbaryl	1	0.5	Acephate	1	0.5
	Dacthal	5	2.5	Aldicarb	3	1.5
	Demeton	3	1.5	Disulfoton	1	0.5
	Endosulfan	1	0.5			
	Methomyl	1	0.5			
	Mevinphos	1	0.5			
	Naled	4	2.0			
	Permethrin	1	0.5			
Cabbage (295)	Disulfoton	1	0.3	Acephate	1	0.3
	Endosulfan	2	0.7	Diazinon	1	0.3
	Mevinphos	1	0.3			
	Monitor	3	1.0			
	Naled	2	0.7			
	Permethrin	5	1.7			
Carrots (226)	Dichloran	1	0.4	4,4′-DDE	12	5.3
	Linuron	4	1.8	Dichloran	1	0.4
	Trifluralin	77	34			
Celery (200)	Acephate	4	2.0	Propazine	1	0.5
	Chlorothalonil	14	7.0			
	Diazinon	2	1.0			
	Dichlone	1	0.5			
	Dichloran	24	12.0			
	Dyrene	4	2.0			
	Endosulfan	6	3.0			
	Malathion	2	1.0			
	Permethrin	10	5.0			
Cucumbers (147)	Endosulfan	30	20	Carbofuran	1	0.7
	Toxaphene	1	0.7	Dieldrin	9	6.1
				Heptachlor epoxide	6	4.1
Grapefruit (106)	Aldicarb	7	6.6			
	Carbaryl	19	18			
	Chlorpyrifos	1	0.9			
	Methidathion	1	0.9			
	Methomyl	3	2.8			
	Parathion	1	0.9			
Grapes (198)	Captan	34	17	Demeton	1	0.5
	Carbaryl	2	1.0			
	Dichloran	16	8.0			
	Dimethoate	3	1.5			
	Endosulfan	9	4.5			
	Ethion	1	0.5			
	Iprodione	1	0.5			
	Methomyl	10	5.1			
	Vinclozolin	12	0.6			
Greens (153)	Aldicarb	3	2.0	2,4′-DDE	1	0.7

Table 4. ***Continued***

Commodity and (No. of samples)	Compounds below tolerance	No. found	Percent	Compounds with violations	No. F.S.	Percent
	Dacthal	20	1.3	4,4′-DDE	6	3.9
	Diazinon	2	1.3	Endosulfan	2	1.3
	Dimethoate	2	1.3	Mevinphos	4	2.6
	Endosulfan	2	1.3			
	Methomyl	1	0.7			
	Mevinphos	10	6.5			
	Permethrin	6	3.9			
Lemons (139)	Aldicarb	7	5.0	Aldicarb	1	0.7
	Carbaryl	18	13			
	Chlorobenzilate	2	1.4			
	Chlorpyrifos	6	4.3			
	Dimethoate	1	0.7			
	Methidathion	7	5.0			
	Methomyl	4	2.9			
	Parathion	2	1.4			
Lettuce (318)	Cypermethrin	1	0.3	4,4′-DDE	5	1.6
	Dacthal	4	1.3	Diazinon	1	0.3
	Endosulfan	17	5.3	Mevinphos	1	0.3
	Iprodione	1	0.3			
	Malathion	1	0.3			
	Methomyl	2	0.6			
	Mevinphos	4	1.3			
	Permethrin	27	8.5			
Limes (78)	Aldicarb	1	1.3	Permethrin	1	1.3
	Carbaryl	5	6.4			
	Parathion	1	1.3			
Melons (56)	Endosulfan	10	18			
Mushrooms (394)	Chlorthalonil	8	2.0	Aldicarb	1	0.3
Nectarines (45)	Captan	1	2.2			
	Dichloran	17	38			
Onions (429)	Captan	2	0.5	Aldicarb	3	0.7
	Chlorthalonil	2	0.5	Chlorthalonil	3	0.7
	Dacthal	5	1.2	Cypermethrin	1	0.2
	Dichloran	3	0.7	Dichlobenil	1	0.2
	Folpet	1	0.2	Fluchloralin	1	0.2
	Lindane	4	0.9			
	Vinclozolin	1	0.2			
Oranges (220)	Aldicarb	9	4.1	Methiocarb	3	1.4
	Carbaryl	28	13	Chlorpyrifos	1	0.5
	Chlorpyrifos	19	8.7	Methidethion	1	0.5
	Dichlobenil	1	0.5			
	Ethion	4	1.8			
	Methidathion	12	5.5			
	Methomyl	1	0.5			
	Parathion	6	2.7			
	Methiocarb	2	0.9			
Peaches (84)	Captan	4	4.8	2,4′-DDT	1	1.2
	Carbaryl	6	7.1			
	Chlorthalonil	1	1.2			

Table 4. ***Continued***

Commodity and (No. of samples)	Compounds below tolerance	No. found	Percent	Compounds with violations	No. F.S.	Percent
	Dichloran	23	27			
	Parathion	1	1.2			
Pears (71)	Captan	3	4.2	Bayleton	1	1.4
	Endosulfan	3	4.2			
	Ethion	1	1.4			
Peppers (179)	Acephate	4	2.2	Chlorthalonil	1	0.6
	Captan	1	0.6			
	Carbaryl	4	2.2			
	Chlorpyrifos	6	3.4			
	Dimethoate	2	1.1			
	Endosulfan	20	11			
	Ethion	1	0.6			
	Malathion	1	0.6			
	Methomyl	2	1.1			
	Monitor	2	1.1			
	Permethrin	13	7.3			
	Vinclozolin	1	0.6			
Plums (53)	Captan	1	1.9	Methomyl	1	1.9
	Carbaryl	5	9.4			
	Dichloran	15	28			
	Endosulfan	1	1.9			
Potatoes (741)	Aldicarb	54	7.3	4,4′-DDE	9	1.2
	Metolachlor	1	0.1	Aldicarb	1	0.1
				Dichloran	1	0.1
				Dieldrin	2	0.3
				PCNB	1	0.1
Radishes (79)	Dichlorvos	1	1.3	4,4′-DDE	1	1.3
				Dacthal	1	1.3
				Methiocarb	1	1.3
				Mevinphos	1	1.3
				Naled	1	1.3
Spinach (112)	Endosulfan	7	6.3	4,4′-DDE	3	2.7
	Methomyl	1	0.9			
	Permethrin	23	21			
Squash (290)	Chlorothalonil	5	1.7	4,4′-DDE	1	0.3
	Endosulfan	16	5.5	Dieldrin	7	2.4
				Dimethoate	1	0.3
Strawberries (76)	Captan	8	11	Dyrene	2	2.6
	Endosulfan	9	12			
	Ethion	1	1.3			
	Malathion	6	7.9			
	Methomyl	4	5.3			
	Parathion	1	1.3			
	Vinclozolin	14	18			
Tomatoes (207)	Chlorthalonil	3	1.4	Cypermethrin	1	0.5
	Dichloran	5	2.4	Aldicarb	1	0.5
	Endosulfan	1	0.5	BHC	1	0.5
	Monitor	10	4.8	Chlorfenvinphos	1	0.5
	Permethrin	9	4.3	Imidan	1	0.5

Table 4. *Continued*

Commodity and (No. of samples)	Compounds below tolerance	No. found	Percent	Compounds with violations	No. F.S.	Percent
				Chlorpyrofos	1	0.5
Turnips (44)	Chlorpyrifos	1	2.3	Monitor	1	2.3
	Dacthal	4	9.0	Permethrin	1	2.3
	Mevinphos	2	4.5	Dieldrin	1	2.3

Table 5. Ten commodities with the most frequent rates of violation

Commodity	No. of samples analyzed	Percent of samples with residues in violation
Cucumbers	147	10.9
Greens	153	8.5
Turnips	44	6.8
Radishes	79	6.3
Carrots	226	5.8
Squash	290	3.1
Tomatoes	207	2.9
Spinach	112	2.7
Strawberries	76	2.6
Broccoli	203	2.5

Table 6. Ten commodities with the most frequent rates of containing residues with allowable tolerance

Commodity	No. of samples analyzed	Percent samples with residues found below tolerance
Strawberries	76	56.6
Grapes	198	44.4
Peaches	84	41.7
Plums	53	41.5
Nectarines	45	40
Oranges	220	37.3
Carrots	226	36.3
Celery	200	34.5
Lemons	139	33.9
Peppers	179	31.8

Table 7. Pesticide results by category

Category	No. of samples	No. of hits below tolerance	Percent	No. of violations	Percent
Brassica	778	77	9.9	23	3.0
Bulb vegetables	447	18	4.0	9	2.0
Cereal grains	61	0	0	1	1.6
Citrus fruits	583	168	28.8	6	1.0
Cucurbits	651	61	9.4	27	4.1
Fruiting vegetables	459	88	19.2	8	1.7
Leafy vegetables	659	157	23.8	11	1.7
Legume vegetables	116	10	8.6	6	5.2
Pome fruits	408	35	8.6	3	0.7
Root and tuber	1248	145	11.6	41	3.3
Small fruits/berries	281	131	46.6	4	1.4
Stone fruits	195	75	38.5	4	2.1
Tropical/subtropical	309	1	0.3	2	0.6

Table 8. Commodities grouped by category type

Root and tuber		Bulb vegetables		Leafy vegetables	
Artichokes	Potato	Garlic		Asparagus	Lettuce
Beets	Radishes	Leeks		Celery	Parsley
Carrots	Rutabagas	Onions		Escarole	Spinach
Ginger	Turnips				
Parsnips	Yams				
Brassica		**Legume vegetables**		**Fruiting vegetables**	
Broccoli	Cauliflower	Bean sprouts	Soybeans	Eggplant	
Brussels sprouts	Kohlrabi	Beans	Sprouts	Peppers	
Cabbage	Greens	Peas		Tomatoes	
Cucurbit vegetables		**Citrus fruits**		**Pome fruit**	
Cantaloupes	Pumpkins	Grapefruit	Oranges	Apples	
Cucumbers	Squash	Lemons	Tangelos	Pears	
Melons	Watermelon	Limes	Tangerines	Quince	
Stone fruits		**Small fruits/berries**		**Cereal grains**	
Apricot	Peaches	Blueberries	Raspberries	Corn	
Cherries	Plums	Cranberries	Strawberries		
Nectarines		Grapes			
Tropical/subtropical					
Avocados	Papayas				
Bananas	Passion fruit				
Choriomas	Persimmons				
Jicama	Pineapple				
Kiwi	Pomegranates				
Mango	Star fruit				

tain trifluralin (as illustrated in Table 4) at below the EPA tolerance of 1 ppm.

The overall commodity results are categorized in 13 groups as indicated in Table 7. The actual commodity breakdown per category is given in Table 8. Here, the legume vegetables are the most frequent; however, a lesser number of these commodities were taken as compared to cucurbits or brassica, and a small amount of positive residues inflated their percentage values. As an overall summary, the violations are predominated by materials that may already be present in the soil in which crops are being grown.

As illustrated in Table 9, which lists the more predominant residues found, the organochlorine compounds prevail. There is little question that this is because of their stability in the environment.

Conclusions

We believe that the nation's food supply is a safe one. There is no question that the use of pesticides brings a risk of residues; however, in this study we show that risk is minimal. Even so, the general public is interested in having a food supply that approaches zero presence of residues. For this reason, thorough professional analytical testing, such as that provided by this program, may be necessary for several years to come. In this age of residue testing, today's farmers may be faced with paying for the results of improper applications from the past. Also, because of the stability of the materials detected, this situation is not likely to change for some time.

Acknowledgments

The authors thank William Fry, Richard Parker, and the staff at H.E.B. Food Stores for their contribution in this effort.

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Table 9. Frequency of individual residues found

Compound found	Amount	Compound found	Amount
Endosulfan I, II, III	164	Parathion-methyl	12
Dichloran (Botran)	105	Dimethoate (Cygon)	11
Permethrin	100	Diazinon	10
Carbaryl (Sevin)	91	Acephate	10
Trifluralin (Treflan)	79	Parathion	8
Captan	56	Naled (Dibrom)	7
Dacthal	50	Monitor	7
Aldicarb sulfoxide	46	Dyrene	7
Chlorthalonil (Bravo)	41	Heptachlor epoxide	6
Chlorpyrifos (Dursban)	41	Methiocarb (Mesurol)	4
4,4′-DDE	41	Linuron	4
Methomyl (Lannate)	31	Iprodione (Rovral)	4
Vinclozolin (Ronilan)	28	Dimeton (Systox)	4
Aldicarb sulfone	27	BHC (Benzahex)	4
Mevinphos (Phosdrin)	25	Lindane (Gamma-BHC)	3
Dieldrin	22	Cypermethrin	3
Aldicarb (Temix)	22	Disulfoton	2
Methidathion (Supracide)	20	Dichlobenil (Casoron)	2
Ethion	19	Chlorobenzilate (Acaraben)	2
Malathion	14	2,4′-DDE	2

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REGULATORY ANALYTICAL METHODS

Introduction

Regulatory agencies approve and promulgate methods of analysis that regulate commodities in their markets. In many instances, these internally approved methods are not widely disseminated outside the agencies. In order to give a method a wider audience, an agency may submit it for publication in the *Journal of AOAC International*. This "Regulatory Analytical Methods" section in the Journal will provide select methods submitted by an agency. Final decisions to publish a method rests solely with the Editor-in-Chief of the Journal.

The methods published under this section have received approval by a national, state/provincial, or international regulatory agency or body and are used to regulate commodities that fall within the scope of the Journal. Each regulatory analytical method published contains its own introduction that explains the origin, nature, and approval that the particular method has undergone.

Having been approved by the regulatory agency submitting the method, the methods published under this section do not undergo the peer review accorded AOAC Journal articles before being published. These methods are ***not*** AOAC Official Methods of Analysis, since they have not been subjected to the full AOAC collaborative study process.

The intent of publishing these regulatory methods in the Journal is to give them a wider distribution and provide them with a publication reference. It is hoped that this section will be used by regulatory bodies of the world to disseminate their methods to scientists everywhere. They are not, however, meant to supplant the AOAC collaborative study process, and AOAC encourages all regulatory bodies and associated industries to support that process.

Animal Drugs

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The U.S. Food and Drug Administration (FDA) is required by the general safety provisions of sections 409, 512, and 706 of the Federal Food, Drug and Cosmetic Act to determine if each food additive, new animal drug, or color additive proposed for use in food producing animals is safe. The pertinent regulations implementing the statutory provisions are found in 21 CFR Parts 70 and 500.

The sponsor of an application for use of an animal drug is required as part of the approval to submit scientific data to demonstrate that the use of the drug is safe for the animals proposed and any edible food to be used for consumption. To demonstrate the safety of the drug, the sponsor must submit acceptable analytical methods. These methods must be capable of determining and confirming the amount and presence of the animal drug or its metabolites in a variety of matrixes.

FDA typically requires analytical methods for finished pharmaceutical and medicated feed dosage forms and for residues of the drug in edible animal tissues. The sponsor must present data or information that demonstrates the method can perform what it purports according to sound analytical principles. In addition, FDA performs a trial of the method according to strict procedures to assure the method is acceptable. The U.S. Drug Administration laboratories also participate with FDA in testing tissue residue methods.

The types of methods described above, because they are used for regulatory purposes by FDA in its compliance programs, are available for release to the general public. These methods are releasable under the Freedom of Information Act. 21 CFR 514.11(e)(6) specifically permits the public disclosure of assay methods after approval of the animal drug has been published in the *Federal Register*.

Because of the volume and types of feeds containing approved animal drugs and used for food-producing animals, the FDA Center for Veterinary Medicine (CVM) has made it a policy to make available to the public methods for animal drugs in Type A Medicated Articles (premixes) and the corresponding Type B and C Medicated Feeds and tissue residues. CVM is the unit responsible for evaluating the effectiveness and safety of animal drugs.

Before 1973, the methods were incorporated into a manual called the *Food Additives Analytical Manual*. This manual, which is no longer available, included methods for direct and indirect food additives and animal drugs. In 1985, CVM issued a new manual called *Animal Drug Analytical Manual*. This manual, often referred to as ADAM, contains only methods for animal drugs. The 1985 edition contained only 8 methods. The manual is published and distributed by AOAC International. Information for the manual is furnished by CVM.

Since 1985, there have been no updates to ADAM. CVM, in cooperation with AOAC International, is now introducing a new procedure for making methods more readily available. It is the intention of CVM in conjunction with AOAC International to first publish the methods in the *Journal of AOAC International* and then incorporate the same in a later update to ADAM.

Clorsulon

The following methods are approved regulatory methods to determine and confirm residues of clorsulon in cattle.

REGULATORY ANALYTICAL METHODS

Method I. Liquid Chromatographic/Ultraviolet Determination of Clorsulon Residues in Kidney Tissues of Cattle

Co-Edited by: JOHN MARKUS[1]
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Clorsulon is approved for use in cattle as a drench. It is approved for the treatment of immature and adult liver flukes (*Fasciola hepatica*) infestations. The product is not to be used in dairy cattle of breeding age.

The drug is also approved in combination with ivermectin for the treatment and control of various parasites, viz., gastrointestinal nematodes, lungworms, liver flukes, grubs, and mites.

Regulation Information

The drug product was approved for use on March 14, 1985 (1), and listed under 21CFR520.462. Clorsulon is also approved for use in cattle in combination with ivermectin as an injectable product under 21CFR522.1193.

Tolerances: 21CFR556.163

The tolerance for clorsulon in cattle kidney is 1.0 ppm.

Target tissue.—Cattle kidney.

Marker residue.—1.0 ppm clorsulon corresponds to a total residue of 3.0 ppm in kidney.

The safe concentration for total clorsulon residues in uncooked edible cattle tissues are 1.0 ppm, muscle; 2.0 ppm, liver; 3.0 ppm, kidney; and 4.0 ppm, fat.

Tolerances and residue information for ivermectin in cattle can be found in 21CFR556.344(a) and in the Ivermectin methods published in *J. AOAC Int.* (1992) **75**, 757–771.

Method I. Liquid Chromatographic/Ultraviolet Determination of Clorsulon Residues in Kidney Tissues of Cattle

Scope

Clorsulon residues are determined in cattle tissues. The method is applicable to the drug when used alone or in combination.

Received January 14, 1991. Accepted December 4, 1991.
[1] Current address: 1709 Burning Tree Dr. Vienna, VA 22182.

Principle

The tissue sample is treated with hydroxylamine and ammonia and homogenized with ethyl acetate using a Polytron homogenizer. The ethyl acetate is separated and removed by evaporation. The residue, dissolved in an aqueous media with a small amount of acetonitrile present, is first washed with hexane and then absorbed on a Bond-Elut CH column. An aliquot of the acetonitrile eluate is compared by liquid chromatographic (LC)/ultraviolet (UV) detection to a series of standards. See Figure 1 for a diagram of the method.

Limit of Reliable Measurement

The determinative method was validated by analysis of controlled animal samples dosed at 0.5, 1.0, and 2.0 ppm. The data from the validation trial are presented in Table 1.

Apparatus

(All references to commercial apparatus and chemicals in this and the following sections are for descriptive purposes only and do not constitute endorsement or recommendation of a product by the U.S. Food and Drug Administration (FDA) or any U.S. government agency; equivalent products may be substituted.)

(a) *Adapters.*—To connect columns, etc. (Analytichem International No.636001).

(b) *Aspiration system.*

(c) *Balance.*—Analytical, capable of weighing 1 mg accurately (Mettler M5/SA microbalance).

(d) *Balance.*—Capable of weighing 1 and 5 g accurately (Mettler P1200 top loading balance).

(e) *Bottles.*—Squeeze type, 50 mL.

(f) *Ultrasonic bath.*—Sonogen automatic cleaner (Bronson Model 52G or B32).

(g) *Water bath.*—Variable temperature, 40–80°C, equipped with nitrogen lines and disposable glass pipet nozzles for evaporation.

(h) *Ice bath.*—Ice plus water mixed in a 500 mL beaker.

(i) *Centrifuge.*—IEC Model HN-S II with 6 place rotor, IEC No. 950, for 15 and 50 mL centrifuge tubes. The centrifuge should be capable of 2000–2500 rpm and a 700–750 × *g* maximum centrifugal force.

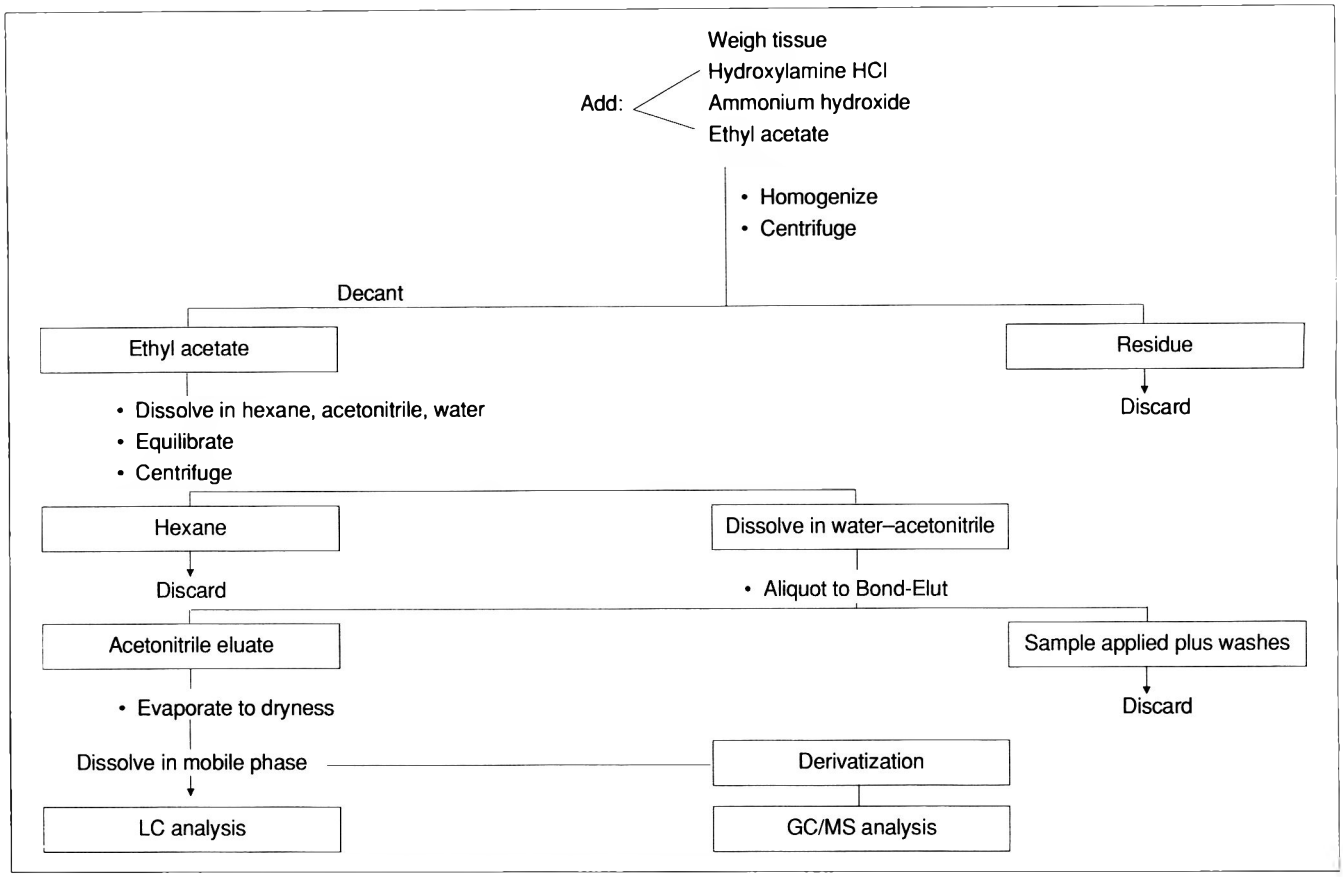

Figure 1. Flow diagram of clorsulon tissue residue method.

(**j**) *Centrifuge tubes.*—Glass, 15 and 50 mL, with polyethylene stoppers.

(**k**) *Columns.*—Bond Elut CH (cyclohexyl), 1000 mg, 6 mL (Analytichem International No. 610406).

(**l**) *Culture tubes.*—Disposable, 13 × 100 mm, borosilicate glass (Fisher).

(**m**) *Depth filters.*—Analytichem International No. 650006.

(**n**) *Dispensing pipetters.*—10 and 20 mL.

(**o**) *Filtration unit.*—Complete with Millipore LSWP02500 filter.

(**p**) *Flask.*—Erlenmeyer, 2 L.

(**q**) *Flasks.*—Volumetric, glass, 50, 100, 500, and 1000 mL.

(**r**) *Freezer.*—Capable of reaching −20°C.

(**s**) *Graduated cylinders.*—250 and 1000 mL.

(**t**) *Homogenizer.*—Polytron (Brinkmann Instruments No. 27-11-200-5) with PTA 10S generator (Brinkman No. 7-11-330-3).

(**u**) *pH Meter.*

(**v**) *Glassine paper.*

(**w**) *Pipets.*—0.2, 1, 5, and 10 mL measuring; 0.2, 0.5, 1, 5, 10, and 25 mL volumetric and disposable.

(**x**) *Pipet bulbs.*—Latex rubber or silicone rubber.

(**y**) *Parafilm.*—American Can Co.

(**z**) *Refrigerator.*—4–5°C.

(**aa**) *Repipet.*—20 mL variable volume (Labindustries).

(**bb**) *Pipettor.*—20 mL glass dispensing head.

(**cc**) *Reservoirs.*—25 mL (Analytichem International No. 602500).

(**dd**) *Shaker.*—Adjustable, 2 speed (Eberbach No. 6010).

(**ee**) *Spatula.*—Stainless steel.

(**ff**) *Syringe.*—glass, 10 and 50 µL.

(**gg**) *Vac-Elut vacuum manifold.*—Analytichem International.

(**hh**) *Vortex mixer.*

(**ii**) *Gloves.*—Disposable PVC or Flexam.

(**jj**) *Tape.*—0.5 in. wide (Time Products, Burr Ridge, IL).

(**kk**) *Liquid chromatograph.*—Beckman-Altex 110A with Valco sample valve with syringe loading sample loop or Waters WISP Autosampler, Kratos-Schoeffel Instruments Spectroflow Model 773 UV absorbance detector, and a 10 mV recorder.

(**ll**) *Pre-column.*—5 µm, 4.6 mm id C_{18} standard Brown-Lee Labs guard column (Spheri-5 RP-18 OD-GU (Rainin Instrument Co.) (**Note**: Replace guard column frequently, especially when pressure buildup and/or peak deterioration is observed.)

Reagents and Solutions

(**a**) *Acetonitrile.*—Distilled in glass (Burdick and Jackson), LC grade (Fisher), or nanograde (Mallinckrodt).

(**b**) *Ammonium hydroxide.*—Concentrated or 29% (Fisher ACS reagent).

(**c**) *Ethyl acetate, methanol.*—Pesticide grade (Fisher).

(**d**) *Hexane.*—Nanograde (Mallinckrodt) or distilled in glass (Burdick and Jackson).

(**e**) *Hydrochloric acid.*—1M (J.T. Baker, Dilut-It); 0.002M: dilute 1.0 mL 1M HCl to 500 mL with deionized water.

(**f**) *Hydroxylamine hydrochloride.*—Analytical reagent (Mallinckrodt) or ACS reagent (Fisher). (**Note**: If the chemical is not finely ground, grind it in a mortar and pestle.)

(**g**) *Sodium bisulfite.*—Reagent grade (J.T. Baker).

(**h**) *Sodium hydroxide.*—50% (w/w), analytical reagent (Mallinckrodt).

(**i**) *Potassium phosphate, monobasic, anhydrous.*—Analyzed reagent (J.T. Baker) or ACS reagent (Fisher).

(**j**) *Water.*—Suitable for LC, such as provided by Millipore Milli-Q system.

(**k**) *Phosphate buffer.*—0.01M, pH 7: weigh 1.36 g potassium phosphate into a 2 L Erlenmeyer flask. Add 1 L Millipore water and stir or swirl to completely dissolve. Use a pH meter to adjust the pH to 7.0 with 50% (w/w) sodium hydroxide. Filter through a Millipore LSWP02500 filter using vacuum. Prepare fresh weekly.

(**l**) *LC mobile phase.*—Mix 800 mL phosphate buffer with 200 mL acetonitrile. Sonicate in an ultrasonic bath for 30 min to degas.

(**m**) *Standards.*—Weigh accurately 3–5 mg standard clorsulon into a 100 mL volumetric flask. Add ca 30 mL distilled water; use ultrasound to effect complete dissolution. Dilute to volume with distilled water and mix. Dilute to the desired concentration levels with mobile phase for LC analysis. Thoroughly mix and run a standard set for each group of unknown samples. Select the absorbance scale so that the peak height for the highest concentration is 70–90% of full scale deflection. Run standards before and after unknown sets. Reasonable checks of at least ±5% should be obtained. Store standard solutions in a refrigerator (ca 4°C). Warm to room temperature before use each day.

Analysis

Safety precautions.—No particularly dangerous chemicals are used in the method. Only the homogenization procedure needs special attention. Because this homogenization uses nonaqueous solvents, the homogenization vessel should be surrounded by ice water to help prevent the solvent from evaporating into the working mechanism of the homogenizer. The operator, who should be wearing suitable eye protection, should be screened from the apparatus.

Isolation procedures for tissues.—For homogenization and extraction, weigh onto a series of glassine paper pieces 1 g finely ground, solid hydroxylamine hydrochloride for each sample to be analyzed. Into each of a series of 50 mL centrifuge tubes, add 20 mL of ethyl acetate by dispensing pipetter. Prepare one tube for each sample to be run (Tube 2).

Weigh accurately 5.0 g of tissue sample into a 50 mL centrifuge tube (Tube 1). To each individual tissue sample, in rapid order as given, add: (*1*) one weighed hydroxylamine hydrochloride sample, add sample in one spot down one side of the tube on top of the tissue; (*2*) any fortification in water in a small volume (0–0.2 mL), carefully add the fortification on the top of the hydroxylamine; (*3*) 0.1 mL 29% ammonia by graduated pipet; and (*4*) 20 mL ethyl acetate by dispensing pipetter.

With the tube surrounded by an ice-water bath, homogenize the tissue–ethyl acetate mixture (Tube 1) at a setting of 6 to 7 until a homogeneous mixture is obtained and the tissue is thoroughly homogenized. A 15–20 s homogenization is usually sufficient. The time should not exceed 30 s. Perform the above steps for one sample at a time.

Scrape any loose tissue pieces into the centrifuge tube using a spatula and rehomogenize briefly with the corresponding Tube 2 (5–10 s is usually sufficient). The second homogenization with clean solvent is intended to recover any clorsulon remaining on the probe after the first homogenization. Between the Tube 2 rinse and the next sample, clean the probe by homogenizing briefly in turn with methanol, water, and ethyl acetate and wiping off with a clean paper towel.

Cap Tube 1 and shake 30 min on low speed on a shaking machine with the tubes lying flat, with the length of the tube oriented in the direction of the shaking.

Centrifuge Tube 1 at full speed for 5 min and decant the clear supernatant into a third 50 mL tube (Tube 3). Stopper and shake the corresponding Tube 2. While still shaken up, pour contents of Tube 2 into Tube 1, and use the vortex mixer to thoroughly break up the tissue plug. Stopper and reshake Tube 1 for 10 min, as before. Centrifuge Tube 1 for 5 min and again decant the supernatant liquid, combining the ethyl acetate phases in Tube 3. Evaporate to an oil that strongly resists further evaporation in a water bath (60 to 70°C) using a nitrogen flush.

Hexane/HCl–acetonitrile wash.—While the oil is still warm and molten, add, by dispensing pipetter, 10 mL hexane and swirl to dissolve. Add, by 5 or 10 mL graduated pipet, 1.0 mL acetonitrile, stopper, and use ultrasound to dissolve any clorsulon residue in the acetonitrile. A 30 s ultrasound treatment is sufficient. Add, by pipet, 25.0 mL 0.002M hydrochloric acid and repeat the ultrasound treatment. Shake by hand 1 min and centrifuge 10 min. (**Note**: The upper phase and interface can be aspirated to waste at this time if the analyst feels such aspiration is helpful for the subsequent step. If aspiration is used, retain sufficient lower phase for the subsequent 20 mL pipetting.)

Bond-Elute chromatography.—Prepare the Bond-Elut columns just before use as follows: Rinse the column with one column volume of methanol from a squeeze bottle, followed by a 2× column volume of distilled water using a squeeze bottle. Rinse by placing the column on the vacuum manifold (Vac-Elut), adding solvent (methanol or water), and applying low vacuum to slowly suck solvent through the column (pressure = 10 in. Hg). Adjust the vacuum using the needle valve on the manifold or the main valve. Release the vacuum slowly and carefully between applications of solvent. Rinse depth filters twice with ca 6 mL of water and partially dry by sucking air through them for 1 min. Do not unduly extend the drying period or the column will be too dry to absorb the clorsulon. Place an assembly composed of a clean reservoir on top of a rinsed CH Bond-Elut column onto the Vac-Elut, connecting the parts with blue adopters. If a Vac-Elut manifold is not available, individual column setups can be prepared and used by attaching to a vacuum flask through a one-hole stopper.

Pipet exactly 20.0 mL of the essentially clear bottom phase (avoiding solids) into the reservoir of a Bond-Elut column

Table 1. Summary data (ppm) for clorsulon determinative assay

Sample	Merck	FDA, CVM	FDA, Baltimore	FDA, Denver	USDA, Athens
Control No. 245	0.0	0.0	0.0	0.0	0.0 0.0
0.5 ppm fortified					
1	—	0.49	0.436	0.536	0.59 0.61
2	—	0.51	0.454	0.527	0.555 0.53
3	—	0.48	0.499	0.536	0.517 0.539
4	—	0.48	0.459	0.527	0.404 0.467
5	—	0.51	0.439	0.527	0.557 0.441
Av. (rec., %)	—	0.494 (97)	0.457 (93.3)	0.530 (104)	0.521 (104)
CV, %	—	4.1	5.4	1.0	12.6
1.0 ppm fortified					
1	—	0.91	1.002	1.01	1.01 1.06
2	—	0.94	1.004	0.858	0.875 0.929
3	—	0.975	1.064	1.07	1.37 0.862
4	—	1.01	0.995	1.11	0.779 0.966
5	—	0.975	1.117	1.086	0.936 0.956
Av. (rec., %)	—	0.963 (96)	1.036 (105.7)	1.026 (105.6)	0.974 (97.4)
CV, %	—	4.0	5.06	9.9	16.4
2.0 ppm fortified					
1	—	1.95	2.16	1.93	1.94 1.96
2	—	1.91	1.99	2.05	1.95 1.90
3	—	1.93	1.95	2.12	1.91 1.78
4	—	1.95	1.90	2.18	1.61 1.84
5	—	1.97	1.78	2.00	1.87 2.08
Av. (rec., %)	—	1.942 (97)	1.96 (99.8)	2.056 (105.7)	1.884 (94.2)
CV, %	—	1.2	1.1	4.8	6.6
Dosed No. 246 Merck					
1	1.04	1.03	1.15	1.056	1.04 1.04
2	1.06	1.05	1.19	1.043	1.03 1.04
3	1.06	1.10	1.11	1.040	0.93 0.96
4	1.09	1.05	1.21	1.031	1.00 1.07
5	1.04 1.01	1.05	1.19	1.040	0.97 1.12
Av., ppm	1.041	1.056	1.17	1.042	1.02

setup without vacuum. Carefully apply vacuum to slowly load the sample onto the column, eventually increasing the vacuum to ca 10 in. Hg. The column effluent should look very much like the sample applied. If the filter plugs with solids, gently scrape the filter surface using a disposable pipet to resuspend the solids. After all the solution has passed through the system, rinse the reservoir twice with ca 5 mL portions of distilled water from a squeeze bottle with vacuum continued.

Allow the first wash to pass through the system completely before the second is started. Remove the reservoir and repeat washings of the filter. Continue washings until the eluate is clear and essentially colorless. One or 2 washings are usually sufficient.

Remove the filter and rinse the column with an additional 3 mL water. Dry the column by continuing to pull air through after apparent dryness for up to ca 1 min. Slowly release the vacuum and add 4 mL acetonitrile to the column by repipet. Collect the acetonitrile in a 13 × 100 mm test tube by slowly reapplying vacuum up to 10 in. Hg. Make sure all of the acetonitrile has been eluted before slowly releasing the vacuum again. DO NOT REMOVE columns from the system while the vacuum is still on or apply too much vacuum or splashing will occur.

Evaporate the acetonitrile using a nitrogen purge and a 60–70°C water bath. Increase the purge gradually as the volume decreases to avoid splashing. The fully dried residue generally should have a slight amount of scum remaining but no liquid.

Isolation procedures for plasma.—Weigh ca 200 mg solid sodium bisulfite into a series of clean 15 mL centrifuge tubes. Pipet 5.0 mL plasma sample into a tube. Shake briefly (20–30 s) to dissolve the bisulfite. Let stand at room temperature 25 min. Pour ca 13 mL ethyl acetate into the tube, stopper, and shake vigorously 1 min. Centrifuge 5 min. Transfer the upper ethyl acetate layer to a clean 15 mL tube. Avoid transferring of

the lower layer. Blow off the ethyl acetate layer at 60–70°C using a nitrogen purge. Break the residue plug by mixing on a vortex mixer. Add ca 13 mL ethyl acetate to the residual plasma and repeat the extraction. Blow off the ethyl acetate to dryness or as near to dryness as possible. (*CAUTION*: Do not delay any of the steps for more than 1 h.)

Add 1 mL hexane from a repipet and vortex 5–10 s. Add 4 mL more of hexane and 3 mL acetonitrile. Vortex 30 s. Shake 1 min by hand and centrifuge 5 min. Discard the upper hexane layer with a disposable pipet, avoiding removal of any of the lower layer. Repeat the washing with a second 5 mL portion of hexane. Evaporate the acetonitrile to dryness using a nitrogen flush at 50–60°C.

Isolation procedures for fat.—Treat the sample exactly as for other tissues except that the centrifugations of the ethyl acetate homogenate should be 10 min each. Use 30 mL ethyl acetate for the first homogenization; decant through a glass wool plug on a funnel as a filter; after the first 2 ethyl acetate extracts are about half blown off, make a third 20 mL ethyl acetate extract and combine with the partially blown down initial 2 extracts. After the third extract passes through the glass wool, squeeze out the liquid in the wool with a spatula into the centrifuge tube collector. The residue from the fat ethyl acetate extractions should be 4–5 mL oil.

Determinative Procedures

LC conditions.—Column, 25 cm × 4.6 mm id Alltech C_{18}; mobile phase, phosphate buffer– 0.01M acetonitrile pH 7 (8 + 2); column temperature, 32°C; flow rate, 2.0 mL/min at 1000 psi (range of 500–2500 psi is acceptable); retention time, ca 18 min; wavelength, 265 nm; absorbance scale, variable down to 0.005–6 AUFS. (**Note**: The effectiveness of the LC system is judged by running clorsulon standards. If the peak height, peak symmetry, etc., deteriorate, the precolumn should be replaced or the system investigated.)

Analysis of samples.—Dissolve the oil or residue in 0.5 mL mobile phase using a Vortex mixer and ultrasound. A 30 s ultrasound treatment is sufficient. Other suitable volumes may be used depending on the expected concentrations of the samples. Inject 40 µL each of the standard series and then the sample(s). Repeat injection of the standards after the samples. (**Note**: If the LC determination is delayed, store the samples and standards in the refrigerator [4–5°C]. For long periods of delay, store the samples and standards in a freezer [–20°C]. Cover all samples with parafilm during storage.)

Calculations.—Plot a graph of peak height vs concentration (µg/mL) of the standards. The plot should be linear and go through the origin.

For tissues and fat, calculate ppm as follows:

$$\text{ppm} = \mu\text{g/mL} \times V_1/V_3 \times V_2/W = 1.3 \times \mu\text{g/mL} \times V_1/W$$

where µg/mL = µg/mL of unknown read from the standard curve; V_1 = mL sample dissolved in mobile phase for LC; V_2 = mL HCl–acetonitrile that sample was dissolved in before Bond-Elut = 26; V_3 = aliquot used for Bond-Elut = 20 mL; and W = grams of sample weighed.

For plasma, calculate ppm as follows:

$$\text{ppm} = (\mu\text{g/mL})_s \times V_1/V_2 = 0.2\ (\mu\text{g/mL})_s\ V_1$$

where $(\mu\text{g/mL})_s$ = µg/mL of unknown read from the standard curve; V_1 = mL sample dissolved in mobile phase for LC; and V_2 = mL of plasma sample taken.

Assay stopping places and solution stability.—For samples, only 2 stopping places during the assay are considered good: when the residue is dissolved in the mobile phase and is ready for chromatographic analysis, and when the ethyl acetate is completely blown down. The samples at these stages are stable at refrigerator temperatures at least 4–5 days. The stock solution of the standard in water is stable at least 1 year at 4–5°C. The diluted standard solutions are stable at least 1 week when stored in a refrigerator.

Sample throughput.—A total of 12 assay work-ups per person per 8 h day have been demonstrated to be practical.

Recovery procedures.—Determine recovery by analysis of dosed control animal tissue and comparison of theoretical and experimental concentrations.

Confirmatory procedures.—Confirm the presence of clorsulon by the gas chromatography (GC)/mass spectrometry (MS) procedure described in Method II.

Reliability of the Method

Validation.—The clorsulon methods were tested in 1 U.S. Department of Agriculture (Athens, GA) and 3 FDA (Baltimore, MD, Denver, CO, and Beltsville, MD) laboratories. All laboratories participated in the testing of the quantitative method; whereas only Denver and Beltsville participated in the testing of the confirmatory method (Method II). A dosed animal tissue sample was analyzed by all laboratories, including the sponsor of the drug (Merck & Co.).

Table 1 presents summary data from the validation trial of the quantitative method conducted by the participating laboratories. The laboratories reported no interferences at the retention time for clorsulon in the kidney extracts. Recoveries were all above 90% at all fortification levels. The results were well within the 80–110% range for tolerance levels at or below 1.0 ppm. All coefficients of variation were acceptable.

One laboratory used a fixed wavelength detector (254 nm) for the LC determinative step. That laboratory found no interference at the position of the parent peak. Another laboratory during the testing of the confirmatory method found that a capillary column could be used for the separation and determination of the clorsulon derivative by GC/MS.

Reference

(1) Method was submitted by the applicant, Merck & Co., Rahway, NJ, per requirements for approval of a new animal drug application, NADA 136-742 (Approved: March 14, 1985).

Method II. Gas Chromatographic/Mass Spectrometric Confirmatory Method for Identification of Clorsulon Residues in Kidney Tissues of Cattle

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Clorsulon is approved in cattle as a drench. It is approved for the treatment of immature and adult liver flukes (*Fasciola hepatica*) infestations. The product is not to be used in dairy cattle of breeding age.

The drug is also approved in combination with ivermectin for the treatment and control of various parasites, viz., gastrointestinal nematodes, lungworms, liver flukes, grubs, and mites.

Regulation Information

The drug product was approved for use on March 14, 1985 (1), and listed under 21CFR520.462. Clorsulon is also approved for use in cattle in combination with ivermectin as an injectable product under 21CFR522.1193.

Tolerances: 21CFR556.163

The tolerance for clorsulon in cattle kidney is 1.0 ppm.

Target tissue.—Cattle kidney.

Marker residue.—1.0 ppm clorsulon corresponds to a total residue of 3.0 ppm in kidney.

The safe concentration for total clorsulon residues in uncooked edible cattle tissues are 1.0 ppm, muscle; 2.0 ppm, liver; 3.0 ppm, kidney; and 4.0 ppm, fat.

Tolerances and residue information for ivermectin in cattle can be found in 21CFR556.344(a) and in the Ivermectin methods published in *J. AOAC Int.* (1992) **75**, 757–771

Received January 14, 1991. Accepted December 4, 1991.

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Method II. Gas Chromatographic/Mass Spectrometric Confirmatory Method for Identification of Clorsulon Residues in Kidney Tissues of Cattle

Scope

Clorsulon residues are confirmed in cattle kidney tissues.

Principle

Beef kidney tissue containing clorsulon residue is prepared as in the liquid chromatographic (LC) determinative assay (Clorsulon Method I) and then derivatized and purified for gas chromatography (GC)/mass spectrometry (MS) analysis.

The tissue sample is treated with hydroxylamine and ammonia and homogenized with ethyl acetate using a Polytron homogenizer. The ethyl acetate is separated and removed by evaporation. The residue, dissolved in aqueous media with a small amount of acetonitrile present, is first washed with hexane and then absorbed on a Bond-Elut CH column. The acetonitrile eluate is reconstituted in LC mobile phase and an aliquot removed for derivatization by methyl iodide.

The resulting pentamethyl derivative is extracted into hexane and further purified by hexane–water partitions. The hexane extracts are evaporated under nitrogen, and the residue is redissolved in ethyl acetate for analysis by GC/electron impact-mass spectrometry (EI-MS) with multiple ion detection (MID) of the pentamethyl derivative of clorsulon.

Aqueous solutions of clorsulon standard are derivatized, prepared concurrently with the samples, and used to calibrate the GC/MS system.

Limit of Reliable Measurement

The method confirms clorsulon at levels of 0.2 ppm.

Apparatus

(All references to commercial apparatus and chemicals in this and the following sections are for descriptive purposes only and do not constitute endorsement or recommendation of

a product by the U.S Food and Drug Administration or any U.S. government agency; equivalent products may be substituted.)

The following equipment is required in addition to that listed in the *Apparatus* section of Clorsulon Method I, *J. AOAC Int.* (1992) **75**, 937–941.

(**a**) *GC/MS system.*—Finnigan Model 9610.

(**b**) *GC/MS column.*—3 ft (91 cm) × 2 mm id 3% SP2100 on Supelcoport (80–100 mesh).

(**c**) *MS system.*—Finnigan Model 4500 with Incos data system, operated in EI and MID modes.

Reagents and Solutions

The following reagents and solutions are required in addition to those listed in the *Reagents and Solutions* section of Clorsulon Method I.

(**a**) *Methylene chloride.*—Pesticide grade (Fisher) or distilled in glass (Burdick and Jackson).

(**b**) *Methyl iodide.*—Aldrich.

(**c**) *Tetrabutyl ammonium hydroxide (TBAH).*—Electrometric grade (Southwestern Analytical Chemical).

(**d**) *Water.*—Double distilled.

(**e**) *TBAH, 0.4M.*—Dilute 20 mL 1M tetrabutyl ammonium hydroxide to 50.0 mL with double distilled water.

(**f**) *Methyl iodide solution.*—Dilute 1.6 mL methyl iodide to 50.0 mL in a volumetric flask with methylene chloride. Prepare just before use. (**Note**: See *Safety precautions* at the beginning of the *Analysis* section.)

(**g**) *Standards.*—Standards are prepared initially as in the LC determinative assay, Clorsulon Method I. Weigh accurately 3–5 mg standard clorsulon into a 100 mL volumetric flask. Add ca 30 mL distilled water, and use ultrasound to effect complete dissolution. Dilute to the mark with distilled water and thoroughly mix. Dilute this water solution with mobile phase to the highest standard concentration (E) for GC/MS analysis.

Dilute the highest standard accurately: D, 0.8 > 1; C, 0.6 > 1; B, 0.4 > 1; and A, 0.2 > 1.

A convenient method of dilution uses 1 mL graduated pipets, adding 0.8 mL (E) to 0.2 mL mobile phase, etc.

Thoroughly mix and run (E–A) as a standard set for each group of unknown analyses. Prepare standards to be derivatized concurrently with samples. The concentration of E should be greater than the anticipated concentration of the samples. If a wrong estimate is made, adjust the sample volume to fit the standard curve. For example, if samples to be investigated are anticipated to be ca 0.8 ppm and the (E) standard is to represent 1.0 ppm, then for the standard and samples to be in the same final volume, the concentration of the (E) standard should be 1.54 μg/mL at derivatization:

$$\mu g/mL = 1 \text{ ppm} \times V_2/V_1 \times V_4/V_3 \times W = 1.54 \ \mu g/mL$$

where V_1 = mL mobile phase that sample was dissolved in for LC or before derivatization = 0.5 mL; V_2 = mL of sample taken for derivatization = 0.2 mL; V_3 = mL HCl–acetonitrile that sample was dissolved in before Bond-Elut chromatography = 26 mL; V_4 = aliquot used for Bond-Elut chromatography = 20 mL; W = g sample used = 5 g; and ppm = μg/g.

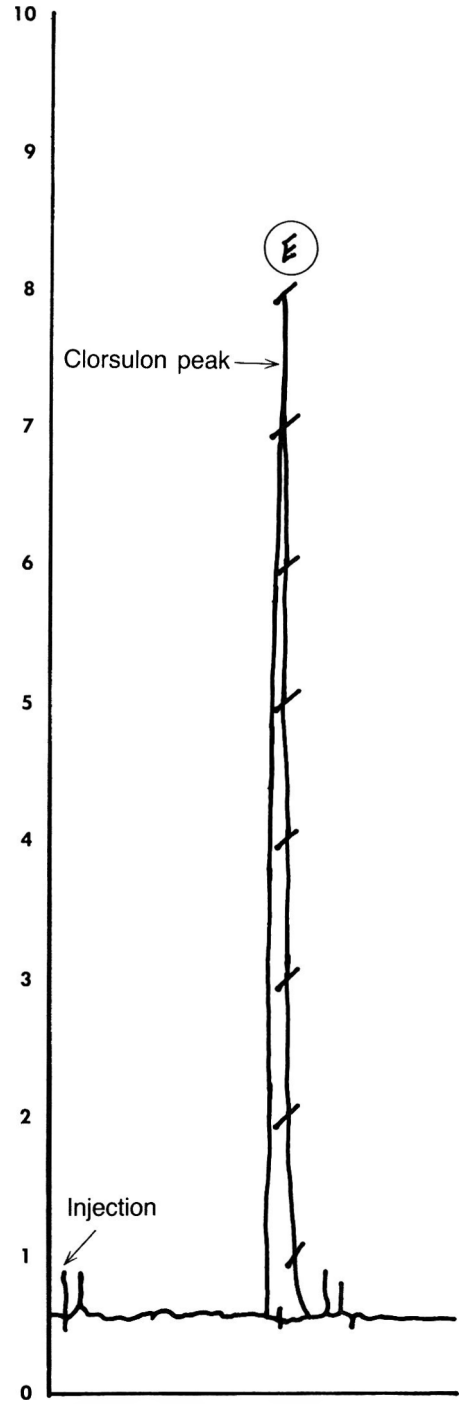

Figure 1. Gas chromatogram of clorsulon standard.

Analysis

Safety precautions.—Special attention to safety is required for homogenization and derivatization. (*1*) *Homogenization*: See *Safety precautions* given in Clorsulon Method I. (*2*) *Derivatization*: Methyl iodide is a poison and suspected carcinogen. To minimize exposure, perform all operations in the derivatization steps in a properly operating fume hood. Use personal protective equipment including gloves, goggles, and a lab coat. Dispose of protective equipment properly after use, or place in proper units for cleaning. Methyl iodide is suffi-

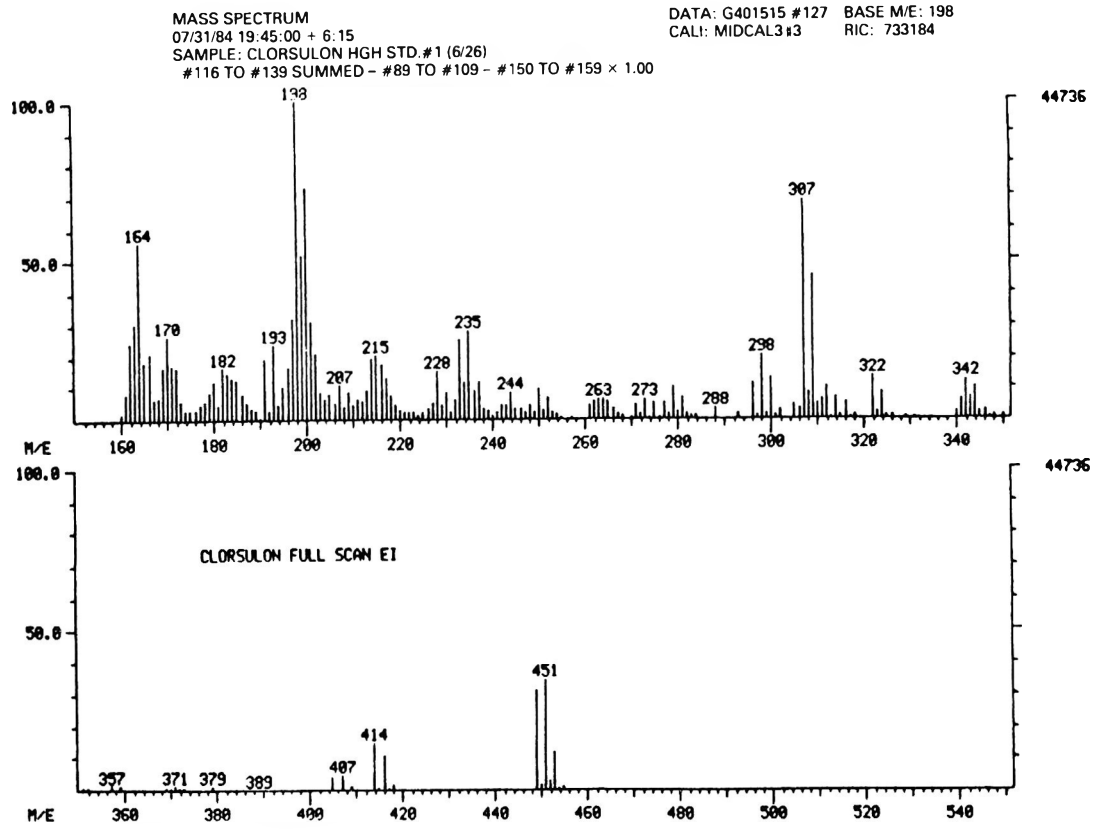

Figure 2. EI mass spectrum of pentamethylchlorsulon.

ciently volatile so that after the 2 evaporation steps in the derivatization procedure, the sample should contain no significant residues and may be treated with the normal care for volatile samples.

Isolation procedures.—Process samples through evaporation to full dryness in the 60–70°C water bath as described in Clorsulon Method I, *Analysis, Isolation procedures for tissues, Bond-Elut chromatography.*

Derivatization.—Add exactly 0.5 mL mobile phase and dissolve using ultrasound and vortex mixing. A 30 s ultrasound treatment is sufficient. Larger dissolution volumes can be used for relatively high expected results. Transfer an aliquot (0.2 mL) from the LC-ready sample to a 15 mL centrifuge tube with tightly fitting stopper. Add mobile phase (0.8 mL) to make the sample volume 1.0 mL. Vortex briefly to assure mixing. Pipet 1.0 mL 0.4M TBAH into each tube containing 1 mL sample or standard solution. Briefly shake this 2 mL aqueous solution, and then add 4.0 mL 0.5M methyl iodide in methylene chloride, freshly prepared just before addition.

(**Note**: Exercise appropriate caution when handling methylene chloride and methyl iodide, which are poisons and suspected carcinogens. Use personal protective equipment, including gloves, goggles, and lab coat. Conduct the operation in a fume hood.) Stopper, tape tightly, and place tubes on the table shaker (set at 100 strokes/min) for shaking overnight (16 h). On the next day, allow the tubes to come to room temperature after removing from the shaker, and then add 5 mL hexane to each tube, using a dispensing pipettor. Stopper, shake 2 min by hand, and centrifuge 5 min at full speed. Transfer the top (organic) phase to another 15 mL centrifuge tube, and re-extract the aqueous phase with an additional 5 mL hexane, repeating the 2 min hand mixing and centrifugation of the stoppered tube.

Combine organic extracts and evaporate to dryness under a stream of nitrogen using a water bath (40°C). The remaining residue is a white solid that first precipitates when the 2 extracts are combined. Partially dissolve the solid in 3.0 mL double distilled water by adding ca 1.5 mL of the water, vortex for a few seconds, and then add the remainder of the water. Add 5 mL hexane using the dispensing pipettor and then stopper. Ultrasonicate 1 min, or as necessary, to dissolve the remaining solid; vigorously shake 2 min by hand and centrifuge 5 min.

Transfer the upper (hexane) phase to another 15 mL centrifuge tube and re-extract the aqueous phase with an additional 5 mL hexane. Combine the second hexane extract with the first and evaporate to dryness under a stream of nitrogen, using a water bath (40–50°C). Redissolve the residue in a volume of ethyl acetate appropriate for GC/MS analysis (e.g., 0.1 mL), using 1 mL ethyl acetate to rinse the sides of the tube. Evaporate again, and then redissolve in a precise volume.

Determinative Procedures

GC operating conditions for GC/MS.—Helium carrier gas flow rate, nominal 30 mL/min; column oven temperature, 240°C; injector temperature, 250°C; and retention time of pentamethylclorsulon, 8 min.

Table 1. Regions of multiple ion detection of pentamethylclorsulon

Region (amu)	Notable ions (amu)	Fragment
449–453	449, 451[a], 453	Parent, Cl isotope cluster
414–416	414, 416	Loss of Cl
341–344	342, 344	Loss of $SO_2N(CH_3)_2$
307–311	307[a], 309	Loss of Cl, $SO_2N(CH_3)_2$
296–300	296, 298, 300	Loss of $SO_2N(CH_3)_2$, $N(CH_3)_2$
233–235	233, 235[a]	Loss of $2(SO_2N(CH_3)_2)$
198–202	198[a], 200	Loss of Cl, $2(SO_2N(CH_3)_2)$

[a] Ions used in identification.

MS operating conditions for GC/MS.—Separator oven, 280°C; transfer line temperature, 265°C; manifold temperature, 100°C; ionization temperature, 140°C; electron multiplier voltage, 1440 V; electron energy, 70 V; emission current, 0.30 mA; and 7 regions with a total of 30 amu monitored in MID mode, with a total scan time of 2.8 s: 198–202, 233–235, 296–300, 307–311, 341–344, 414–416, and 449–453.

Data acquisition.—2.5 min after injection.

Analysis of samples.—To obtain maximum sensitivity, calibrate and tune the MS system with the calibration gas, optimizing the unit at masses 219, 314, and 414. Depending on instrument use, perform this calibration at least weekly. Check the operation of the GC/MS system with injections of standards. Inject 5 µL volumes of standards and samples, and repeat injections to verify reproducibility. Figure 1 shows the chromatogram of a clorsulon standard.

Calculations.—Do a linear regression analysis of the reconstructed ion chromatogram (RIC) peak area vs ppm for the standards. Use the RIC peak area for each unknown sample and the linear regression analysis to determine the ppm for the unknowns uncorrected for assay volume differences. Correct the ppm for the unknown for assay volumes as follows:

$$\text{ppm} = ppm_u \times V_u/V_s \times 1/F$$

where ppm_u = ppm uncorrected; V_u = volume of unknown dissolved in mobile phase; V_s = volume standard dissolved in mobile phase; and F = fraction of the original 5 g sample (determinative assay) in the volume V_u, if not calculated into the standards.

Recovery procedures.—Determine recovery by analysis of fortified samples prepared for LC analysis.

Confirmatory procedures.—No additional confirmatory procedures have been provided.

Reliability of the Method

Validation.—To demonstrate the specificity of the confirmatory assay, kidney samples from control cattle were analyzed. None was found to have detectable levels of clorsulon. Little or no response was observed for the 4 significant ions or for the sample responses, and the ions did not have the appropriate ratios of intensities.

Control samples were fortified with clorsulon at 0.98 ppm. All fortified kidneys had appropriate ratios and quantifiable levels with an average recovery of 97%. Kidney samples from cattle dosed with clorsulon (2 days postdose) were assayed. All samples could be positively identified as containing clorsulon, and the quantitation was in good agreement with the determinative assay.

Discussion

EI-MS with packed column GC was chosen for its simplicity, ruggedness, and broad range of application. Packed column GC provides excellent capacity with good resolution to separate the subject compound from potential interferences. MID makes use of the mass spectrometer as a selective chromatographic detector, monitoring over time the production and intensity of selected characteristic ions. The appearance of these ions at the proper GC retention time and with the appropriate relative intensity for the compound confirms the presence of that compound in the sample.

The pentamethyl derivative of clorsulon is made to improve the GC of the compound. Even though underivatized clorsulon can be determined by LC (Clorsulon Method I), it is not readily amenable to GC. Figure 1 shows that the GC peak of the derivative is sharp and reasonably symmetrical.

The full scan EI mass spectrum of pentamethylclorsulon is shown in Figure 2. For MID, 7 characteristic regions of ions are monitored, as summarized in Table 1. Most ions are fragments formed from the loss of chlorine or the sulfonamide moiety. The ratios of intensities are calculated for 3 ions (m/z = 307, 235, and 451) relative to the base peak (m/z = 198). These ratios, in comparison with standards near the tolerance level (1 ppm), characterize the presence of clorsulon.

Confirmation of the presence of clorsulon in the sample requires the production of each of the 4 significant ions (m/z = 198, 235, 307, and 451), with peak ratios matching those for standards within 10–15% for the 235 and 307 ions and within 40–50% for the 451 ion. The ratio of 451 to 198 displays some concentration dependence near the detection limit for 451 (approximately 0.2 ppm). When only standards near the tolerance limit are employed, the 451 peak ratio of the unknown should be within 10–15% of the standard. The 451 ion is used for identification despite its limited sensitivity because it is the most abundant isotope of the parent ion and is very characteristic of clorsulon.

GC/MS quantitation is based on the peak area of the RIC at the retention time of clorsulon using regression analysis of area vs concentration for a series of standards. The detection limit using the RIC is below 0.2 ppm.

Some work using GC/MS with negative chemical ionization (NCI) was performed before this study. Clorsulon, with 3 chlorine atoms, is a good candidate for NCI, with the pentamethyl derivative yielding characteristic ion clusters at *m/z* 406, 408 (loss of $N(CH_3)_2$) and *m/z* 413, 415 (loss of Cl). The detection limit of the assay (0.001 ppm) far exceeded the required sensitivity, but the NCI technique was not pursued further due to a lack of instrument availability.

Reference

(1) Method was submitted by the applicant, Merck & Co., Rahway, NJ, per requirements for approval of a new animal drug application, NADA 136–742 (Approved: March 14, 1985).

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(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. VanVunakis & J.J. Langone (Eds), Academic Press, New York, NY, pp. 104–142

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

OFFICIAL METHODS REFERENCE

(1) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, secs **29.070–29.072**

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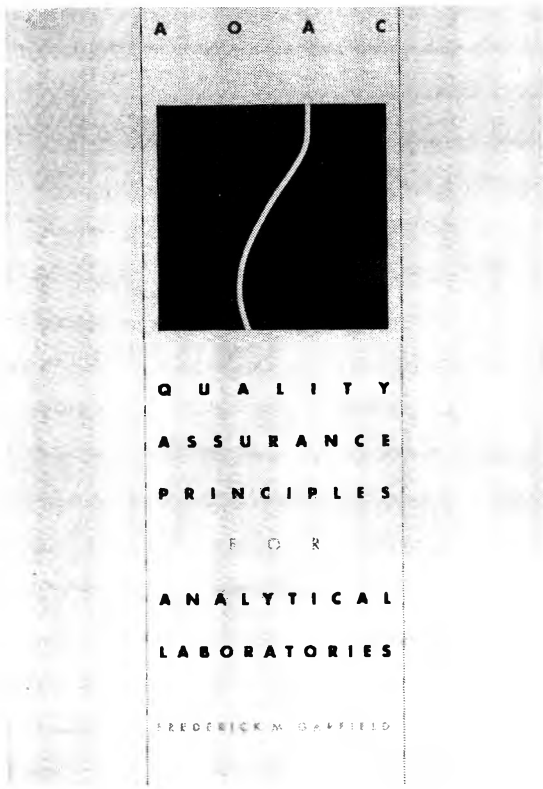

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