ISSN 0004-5756

A O A C

SEPTEMBER 1982
/VOL. 65, NO. 5

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS JOURNAL

JANCA2 65(5) 1039–1296 (1982)

How To Uncover The Hidden Costs Of Chromatography.

Do you realize how much lower-purity HPLC solvents might be costing you? Consider the frequent consequences.

- **The *cost* to repeat separation procedures because of solvent artifacts** — this wastes analytical labor and valuable instrument time.
- **The *cost* to regenerate a water deactivated column** — this causes instrument "downtime" which requires additional chromatographs to perform the same amount of work.
- **The *cost* to wash off a residue-laden column** — the residue decreases column efficiency.
- **The *cost* of replacing columns, filters and check valves clogged with particles** — this can increase costs from $200-800.
- **The *cost* of isolating residue from preparative peaks** — this may cause additional intermediary separations to remove unwanted contaminants.
- **The *cost* of maintaining an additional inventory of several "grades" of purity (HPLC, GC, Spectro, etc.)** — the quality of high purity solvents deteriorates while on a shelf.
- **The *cost* to frequently evaluate the less uniform solvents** — this diverts attention away from productive analytical time.

Avoid these "hidden costs" and time-consuming effects from using lower purity solvents. Switch to B&J Brand High Purity Solvents. Because, when it comes to *cost and performance*, there's no comparison. B&J Brand is your best value.

Sure, less pure solvents might be cheaper. But why jeopardize your chromatography results when you use them? In the long run, lower purity solvents can cost you more because of their inconsistent quality.

Compare for yourself. If you're currently using lower purity HPLC solvents, try the highest purity solvents — B&J Brand. You'll notice the difference right away.

Then do this. Figure out your cost-benefit equation for HPLC chromatography. The answer will be pure and simple. For lower costs and better results, it's B&J Brand High Purity HPLC Solvents.

Write or call today for a free technical bulletin about B&J Brand High Purity HPLC Solvents. We'll also send you our distributor listing so you can conveniently order B&J Brand Solvents from a distributor near you.

Avoid the hidden costs. Switch to B&J Brand High Purity Solvents.

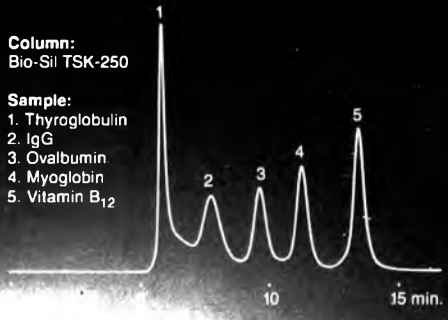
BURDICK & JACKSON LABORATORIES, INC.

1953 South Harvey Street. Muskegon, Michigan U.S.A. 49442 (616) 726-3171

A Subsidiary of Hoffmann-LaRoche Inc

Don't wait forever...

Protein gel filtration in minutes not hours!



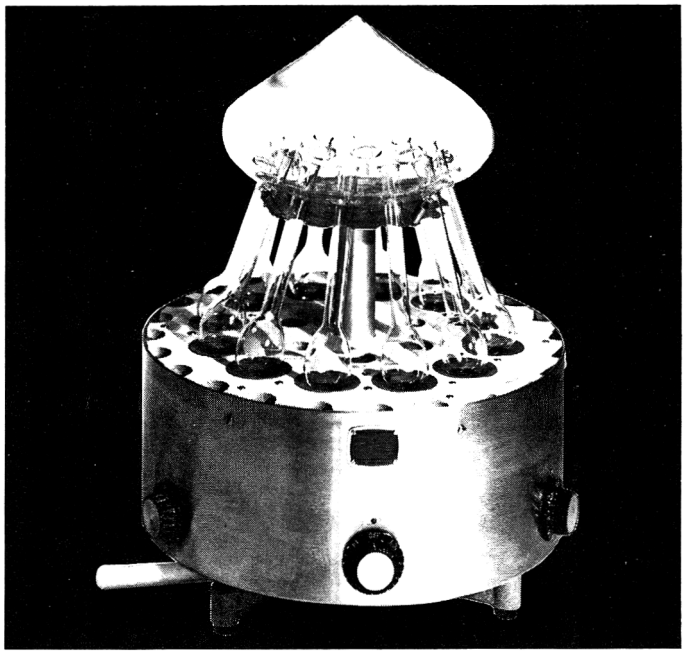
Bio-Rad's HPLC gel filtration system saves hours of time, yet provides separations of proteins or other biopolymers with the same high efficiency as classical methods. For example, the Bio-Rad HPLC separation shown here was completed in only 15 minutes. Using conventional gel filtration, the same separation requires at least 10 hours.

Key system features:
accurate, pulse free solvent delivery • convenient sample injection • sensitive, selectable wavelength • absorbance detection • high efficiency inert columns

Bio-Rad has worked out all the methodology, so you don't have to. And our affordable, modular system is backed by an experienced technical staff ready to assist you with your application. For details, contact:

BIO-RAD

2200 Wright Avenue • Richmond, CA 94804 USA
Phone (415) 234-4130
Also in Rockville Centre, N.Y., Australia, Austria, England, Germany, Italy, Japan, The Netherlands and Switzerland.



This Kjeldahl apparatus has a micro-appetite for nitrogen determinations.

Our unique rotary digestion apparatus is designed for micro and semi-micro determinations* and offers several advantages over conventional designs.

It eliminates troublesome cold-end flasks and fixes all twelve flasks at equal distances ensuring even heating. The entire apparatus occupies only 18 inches of bench space and it can be rotated to position any flask "up-front" or, to agitate flasks. A variable-voltage transformer may be used to expand the temperature range.

A fume hood is available (as shown) to free your laboratory hood for other purposes.

The Kontes Rotary Digestion Apparatus is efficient, compact, and easy to use.

To satisfy your appetite for more product information and applications of this and other Kjeldahl apparatus, contact your Kontes man or send for our product bulletin.

*Application papers include use with plant and biological specimens.

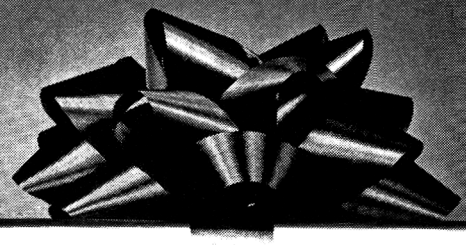
KONTES

SCIENTIFIC GLASSWARE / INSTRUMENTS

Vineland, NJ 08360 (609) 692-8500

Regional Distributors: KONTES OF ILLINOIS, Evanston, Ill. • KONTES OF CALIFORNIA, San Leandro, Calif.

ABC Labs has it all packaged.



Analytical Services

- Pesticide Residues
- Trace Metals (AA-ICAP)
- Drug Residues
- Soil Metabolism
- PCB's in Transformer Oil
- Trace Organic Pollutants
- Water and Waste Water
- Methods Development

Aquatic Toxicology

- EPA Accepted Protocols
- Static Acute Tests
- Time-Independent Acute Bioassay
- Embryo-Larval Tests
- Chronic Investigations
- Bioconcentration Studies
- Effluent Bioassays
- In-House Analytical Support

Instruments

- 1002 Automatic Gel Permeation Chromatograph
- 350B Aquatic Diluter System
- 450 Precision Laboratory Calibrator
- 250 GC Precolumn Venting System
- 150 Filtration System (Asbestos free) for Crude Fiber Analysis

And Straight Talk

We welcome your visit and invite your inquiries. ABC labs has been in business 12 years with a stable support staff. We are in GLP and CGMP compliance, our FDA master file number is 3844, and we have worked with the EPA, USDA, USDI and FDA.

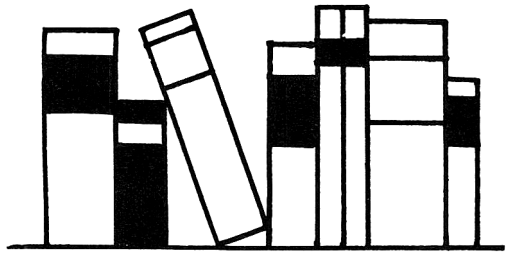
Write or Call for More Information

P.O. Box 1097 • 314/474-8579
COLUMBIA, MISSOURI 65205

abc®

ANALYTICAL
BIO CHEMISTRY
LABORATORIES, INC.

IS YOUR AOAC LIBRARY COMPLETE?



Optimizing Laboratory Performance Through the Application of Quality Assurance Principles—Proceedings of a Symposium. 1981. 160 pp. Softbound. $25 + $3 book post and handling in U.S., $25 + $6 outside U.S. ISBN 0-935584-19-6.
Eleven papers covering setting up and operating a quality assurance program.

FDA Training Manual for Analytical Entomology in the Food Industry. 1978. 184 pp. Looseleaf. $12.50 + $2 book post and handling in U.S., $12.50 + $3 outside U.S. ISBN 0-935584-11-0.
With the aid of this text, organizations can set up their own in-house training.

Infrared and Ultraviolet Spectra of Some Compounds of Pharmaceutical Interest. 1972. 278 pp. Softbound. $12 + $2 book post and handling in U.S., $12 + $3 outside U.S. ISBN 0-935584-04-8.
An expansion of an earlier compilation, with supplements. More than 800 spectra.

Mycotoxins Methodology. 1980. 22 pp. Softbound. $11 + $1 book post and handling in U.S., $11 + $2 outside U.S. ISBN 0-935584-16-1.
Chapter 26 reprinted from Official Methods of Analysis, 13th Edition. Approved methods for natural toxins in many commodities.

Micro-Analytical Entomology for Food Sanitation Control. 1962. 576 pp. Hardbound $30 + $3 book post and handling in U.S., $30 + $6 outside U.S. ISBN 0-935584-00-5.
A training and reference manual for identification of insect debris extracted from foods.

Test Protocols for the Environmental Fate and Movement of Toxicants—Symposium Proceedings. 1981. 336 pp. Softbound. $27 + $3 book post and handling in U.S., $27 + $6 outside U.S. ISBN 0-935584-20-X.
Chemical and biological tests plus methods for interpreting or predicting results through mathematical models.

FDA Bacteriological Analytical Manual (BAM) 5th Ed. 1978. 448 pp. Looseleaf. $25 + $2 book post and handling in U.S., $25 + $5 outside U.S. ISBN 0-935584-12-9.
Provides regulatory and industry laboratories with methods for detection of microorganisms. Updated by supplements.

Mycotoxins Mass Spectral Data Bank. 1978. 60 pp. Softbound. $12 + $2 book post and handling in U.S. and outside U.S. ISBN 0-935584-13-7.
A computer-based compilation of 104 mass spectra with alphabetic and molecular weight listings.

Newburger's Manual of Cosmetic Analysis 2nd Ed. 1977. 150 pp. Softbound. $13 + $2 book post and handling in U.S., $13 + $3 outside U.S. ISBN 0-935584-09-9.
Chromatographic techniques and spectroscopy with analyses for various specific cosmetics.

Statistical Manual of the AOAC. 1975. 96 pp. Softbound. $12.50 + $1 book post and handling in U.S., $12.50 + $2 outside U.S. ISBN 0-935584-15-3.
A do-it-yourself manual for statistical analysis of interlaboratory collaborative tests.

Send check to AOAC, Suite 210-J, 1111 N 19th St., Arlington, VA 22209, 703/522-3032.

JOURNAL of the ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

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

Vol. 65 SEPTEMBER 1982 No. 5

CONTENTS

THE ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, INC.
Printed: The Mack Printing Company, Easton, PA 18042
Published: 1111 N 19th St, Arlington, VA 22209

INFORMATION FOR SUBSCRIBERS AND CONTRIBUTORS

The *Journal of the Association of Official Analytical Chemists* is published by the Association of Official Analytical Chemists, 1111 N 19th St, Arlington VA 22209. The Journal is issued six times a year in January, March, May, July, September, and November. Each volume will contain approximately 1500 pages. Current subscriptions are by volume, $62.00 prepaid in North America and U.S. possessions, and $70.00 to all other countries. Single current issues are $12.00 each ($13.00 foreign). The Journal publishes research articles and reports of the development, validation, and interpretation of analytical methods for agricultural commodities (feeds, fertilizers, pesticides), food (including alcoholic beverages), drugs, cosmetics, colors, the environment, and other areas that affect public health and welfare and are subject to government regulation and control. A limited number of invited reviews on selected subjects are also published.

Manuscripts should be typewritten, double-spaced, and carefully revised before submission; the original and two copies should be submitted to AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. "Instructions to Authors" are available on request from the Editorial Office.

Reprints will be furnished at cost to contributors of articles printed in the Journal. A table showing costs, with an order slip, will be sent with galley proof.

Claim for copies lost in the mails will not be allowed unless received within thirty days of the date of issue for domestic subscriptions or ninety days for foreign subscriptions. Claimants must state that the publication was not delivered at their recorded address. Address requests for replacement copies to AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. For foreign subscribers, replacement of copies of the Journal lost in transit cannot be made without charge because of uncertain mailing conditions abroad.

Change of Address notification should include both old and new addresses, with ZIP numbers, and be accompanied by a mailing label from a recent issue. Allow four weeks for change to become effective. Foreign subscribers should use air mail for notification.

Volumes on microfilm are available from Princeton Microfilm Corp., PO Box 2073, Princeton, NJ 08540.

Postmaster: Forward Changes of Address to AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209.

Official Methods of Analysis of the Association of Official Analytical Chemists

13th Edition

Edited by
William Horwitz
Bureau of Foods
Food and Drug Administration
Washington, DC

Official Methods of Analysis, often referred to as "the bible" by its users, provides research and regulatory chemists and other scientists with reliable methods of analysis for determining the composition of commodities subject to legal control. The reliability of each method adopted by AOAC has been demonstrated by an interlaboratory collaborative study, showing its reproducibility in the hands of professional analysts.

As analytical needs change and as knowledge and techniques advance, new and revised methods are validated by AOAC. These are incorporated into annual updates which are included in the purchase price.

1980, 1038 pp., 55 illus., index, hardbound, Price: $75 + $3 for book post and handling within U.S., $75 + $6 outside U.S., includes 4 yearly updates. ISBN 0-935584-14-5

To obtain this book, please send order and check to
AOAC, 1111 N. 19th St., Suite 210-J, Arlington, VA 22209.

CONTENTS

RADIOACTIVITY

Quantitative Determination of I-131, Cs-137, and Ba-140 in Milk: Collaborative Studies

FREDERICK G. D. SHUMAN, DAVID G. EASTERLY,[1] and EDMOND J. BARATTA[2]
Food and Drug Administration, Bureau of Radiological Health, Rockville, MD 20857

The official method for Cs-137 in milk by gamma-ray spectroscopy was extended to include I-131 and Ba-140. A collaborative study was performed on this method applied to I-131 concentration in cow's milk; the original collaborative study of the method including all 3 nuclides was reviewed. In the I-131 study, 1 aliquot of a milk sample containing 82 pCi/L was sent to each of 60 laboratories for triplicate analyses. From 40 responses, the mean of the reported values was 81.6 pCi/L, indicating a method bias below the 5% statistical detectability limit. Within- and between-laboratory coefficients of variation (CVs) were 7 and 8%, respectively. In the 3-nuclide study, 2 samples were sent to 25 laboratories for triplicate analyses; one sample contained 633, 305, and 515 pCi/L, respectively, of I-131, Cs-137, and Ba-140 and the other contained 98, 52, and 72 pCi/L. For the high-activity sample, within-laboratory CVs were 4-5% for the 3 nuclides and between-laboratory CVs were 4-7%. For the low-activity sample, the corresponding results were 6-9% and 8-16%. The method bias was statistically significant at 95% confidence only for Cs-137 in the high-activity sample; reported results were 3% below the known concentration. The extended method was adopted official first action.

Man-made nuclear reactions, whether in a reactor or a weapons test, introduce various radionuclides into the environment. The levels of certain of these that have made their way into the human food chain through the vehicle of cow's milk must then be determined. This study examines one method used in this determination.

Experimental

The analytical method under study is described in *Official Methods of Analysis* (1). Briefly, a 3.5 L milk sample in a 4 L Marinelli beaker is placed over a NaI(Tl) detector, and a gamma-ray spectrum is acquired. Net count rates are computed for the characteristic emission peaks of I-131, Cs-137, and Ba-La-140, as well as K-40. Mutual interferences among these 4 photopeaks are eliminated by applying a matrix technique to separate the activities of the 4 nuclides. Measurement of 1 standard source of each nuclide provides the matrix coefficients.

METHOD

48.025 ***Principle***

Applicable to ^{131}I, ^{140}Ba, and ^{137}Cs in fluid milk preserved with HCHO. Known vol. is placed in counting vessel positioned over and around right cylinder scintillation crystal detector, NaI(Tl), of multichannel gamma spectrometer. Gamma radiation is counted for given time. Accumulated pulses from selected photon energy range are sepd from other gamma-emitting radionuclides and background radiation by simultaneous equations. ^{40}K is always present as natural contaminant and may contribute counts in 1 or more of photopeak ranges. Mutual interferences among these 4 photopeaks are eliminated by applying matrix technic to sep. activities of the 4 nuclides. Measurement of one std source of each nuclide provides the matrix coefficients.

In special cases, newly formed fission products may be present, e.g., ^{133}I and ^{135}I, which may interfere either thru direct overlapping of photopeaks or by contributing Compton-continuum counts. Such interference may be minimized by waiting for decay of short-lived radionuclides, by addnl counting following decay, or by chem. sepn.

Milk contg known increments of ^{131}I, ^{137}Cs, and ^{140}Ba, detd in triplicate by 25 laboratories,

This report of the Associate Referee, E. J. Baratta, was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

The recommendation of the Associate Referee was approved by Committee E and adopted by the Association. See *J. Assoc. Off. Anal. Chem.* **65**, 385-386 (1982).

Received March 3, 1981. Accepted September 28, 1981.

[1] Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Las Vegas, NV 89114.

[2] Food and Drug Administration, Winchester Engineering and Analytical Center, Winchester, MA 01890.

0004-5756/82/6505-1039-05$01.00

and 2nd milk contg known increment of ^{131}I, detd in triplicate by 40 laboratories, showed results given below (av. of triplicates).

48.026 ***Apparatus***

(a) *Alignment sources.*—Gamma ray energies, at least 1 near ^{137}Cs spectrum, with well known energies and abundance of gamma rays in photopeaks, for alignment. Solid sources, ca 0.1 μCi, are preferred over liq. sources. ^{207}Ba is satisfactory single source with several photopeaks; ^{137}Cs and ^{60}Co are good pair.

(b) *Counter.*—Low level gamma spectrometer consisting of shielded Tl-activated NaI scintillation detector, 4 × 4 in., coupled to multichannel pulse-ht analyzer and readout system.

(c) *Counting vessel (Marinelli beaker).*—Use 3.5 L beaker, Figure **48:01,** for 4 × 4 in. detector. Beaker and lid available from plastic laboratory-ware suppliers such as Bel-Art Products, Pequannock, NJ 07440, No. F26862 for beaker and No. 26872 for lid.

48.027 ***Reagents***

(*Caution: See* **51.075.**)

(a) *Carrier solns.*—10 mg/mL. Prep. solns of CsCl (1.267 g/100 mL), NaI (1.181 g/100 mL), and $BaCl_2.2H_2O$ (1.779 g/100 mL). Store in polyethylene or glass bottles.

(b) *Stock std solns.*—10 000 pCi/mL. Dil. calibrated solns of ^{131}I, ^{140}Ba, and ^{137}Cs to approx. indicated strength.

(c) *Potassium-40 stock std soln.*—1.89 dpm (disintegrations/min) ^{40}K/mg K. Dissolve 240 g KCl (equiv. to 126 g K) in 3 L H_2O in Marinelli beaker and dil. to 3.5 L.

(d) *Calibrating solns.*—For Cs and Ba, add 3–5 mL carrier soln, (a), to 3 L H_2O in Marinelli beaker, mix, add convenient amt of stock std soln, (b), sufficient to reduce counting error to ca 1% when counted within 10–100 min, mix, adjust pH to 3.5–4.5, and dil. to 3.5 L. Prep. I soln similarly, but adjust pH to 8.5.

48.028 ***Determination***

Using alignment sources centered on detector, adjust spectrometer to cover range at least between 0 and 2 meV, in intervals (channels) of 10 or 20 keV. Adjust voltage or gain control so that the 2 gamma photopeaks of std fall in their appropriate channels. Check and adjust alignment daily.

Place Marinelli beaker contg 3.5 L calibrating soln, (d), over detector, and count std for time (10–100 min) sufficient to reduce counting error to ca 1%. Repeat with each calibrating soln and with H_2O. Recalibrate spectrometer yearly or more frequently if gamma-ray resolution changes.

Transfer 3.5 L well mixed milk sample at room temp. into Marinelli beaker, place over detector, and count 100 min or time sufficient to give desired counting statistics.

48.029 ***Calculations***

(a) *Counter efficiency.*—Total individual counts observed in channels of photopeak range for each calibrating soln. Subtract total background count for same photopeak range. Divide net count by counting time in min and amt of radionuclide in pCi, and record cpm/pCi for each.

(b) *Interference coefficients.*—When counting

Amt Nuclide Present, pCi/L	Std Dev. (CV, %)		Bias ± 95% Uncertainty	
	Within Labs	Between Labs	pCi/L	%
^{131}I				
98	6.1 (6.2)	8.2 (8.3)	+0.9 ± 3.7	+0.9 ± 3.8
633	29.0 (4.6)	30.1 (4.8)	+2.3 ± 14.3	+0.4 ± 2.3
^{140}Ba				
72	6.5 (9.1)	11.2 (15.6)	+4.0 ± 4.8	+5.5 ± 6.7
515	19.5 (3.8)	35.8 (7.0)	+7.9 ± 15.8	+1.5 ± 3.1
^{137}Cs				
52	4.7 (9.1)	4.1 (8.0)	+1.3 ± 2.0	+2.4 ± 3.8
305	11.4 (3.7)	13.5 (4.4)	−9.8 ± 6.1	−3.2 ± 2.0
^{131}I				
82	5.6 (6.8)	6.8 (8.3)	−0.4 ± 2.4	−0.5 ± 2.9

Table 1. Collaborative results for radionuclides in milk by gamma-ray spectroscopy (pCi/L): BRH study

	Sample A									Sample B								
Lab.	I-131			Cs-137			Ba-140			I-131			Cs-137			Ba-140		
1	642	640	646	286	287	278	550	547	537	108	105	107	57	49	56	87	83	84
2	617	634	616	296	302	295	504	454	500	103	94	91	57	58	49	69	71	76
3	—	—	—	294	294	294	—	—	—	96	94	96	52	52	55	66	66	64
4	615	557	420	269	304	305	364	498	375	109	111	118	51	52	55	65	93	102
5	607	639	637	300	305	304	488	498	517	85	92	103	51	57	55	67	58	67
6	654	625	659	299	286	300	499	509	493	75	75	80	67	53	58	67	69	68
7	630	633	633	324	322	308	504	506	499	101	99	101	58	61	51	74	73	76
8	640	681	642	289	300	285	509	494	510	103	111	109	52	52	57	72	67	75
9	616	637	615	337	329	322	492	520	517	85	105	88	67	59	60	73	79	82
10	649	646	670	272	300	285	555	561	560	91	107	95	52	59	44	81	82	68
11	535	620	599	245	286	241	593	557	553	69	88	73	48	45	36	112	113	143
12	684	680	580	301	303	276	503	498	494	106	90	82	47	47	45	65	80	79
13	634	650	626	311	306	310	506	510	519	107	107	101	54	50	59	67	77	67
14	661	665	635	302	291	287	575	562	575	106	92	94	60	61	58	74	77	86
15	565	574	561	298	302	298	544	567	569	90	92	94	58	53	53	77	74	81
16	613	656	654	288	309	297	501	538	499	101	103	99	57	49	55	71	68	76
17	617	610	612	295	298	293	534	514	519	104	100	99	50	49	52	69	66	69
18	696	691	677	298	279	292	531	554	537	104	110	112	57	49	51	74	76	71
19	635	626	655	314	304	314	539	561	538	111	96	105	68	58	52	74	68	69
20	668	653	681	291	294	303	484	481	459	108	92	102	51	40	45	69	73	64
21	673	668	615	293	237	269	585	581	570	97[a]	98[a]	—	66	57	44	91	90	91
22	648	652	662	292	294	301	539	547	543	102	100	107	56	55	52	71	69	66
23	670	673	649	270	276	278	556	551	570	105	114	105	40	41	43	83	84	81
24	642	633	626	306	295	303	537	544	543	98	99	95	57	53	54	68	73	75
25	663	670	686	319	292	319	503	510	498	106	111	102	52	55	57	67	66	69
True concn		633			305			515			98			52			72	

[a] Not included in statistical analysis.

std soln of each radionuclide, ^{131}I, ^{137}Cs, ^{140}Ba, ^{40}K, e.g., ^{131}I, ratio of net counting rate in energy range of each of the other radionnuclides to net counting rate in its own photon energy range gives its fractional interfering coefficient for each of the other energy ranges, e.g., ^{131}I ratio of net counting rate in ^{137}Cs energy range to net counting rate in ^{131}I photon energy range gives its fractional interfering coefficient for ^{137}Cs energy range.

Designate counting rate for ^{131}I, ^{140}Ba, ^{137}Cs, and ^{40}K with symbols I, B, C, and K, resp. Designate net counting rates (observed—background) in their resp. photon energy ranges as N_i, N_b, N_c, and N_k, resp. Then, f, fractional coefficients or contributions of nuclide in particular range, is designated by 2 lower case subscripts; first one indicates nuclide contributing counts to energy range (column) and second, nuclide photon energy range (row). The following 4 equations:

$$N_i = I + f_{bi}\,B + f_{ci}\,C + f_{ki}\,K \quad (1)$$

$$N_b = f_{ib}\,I + B + f_{cb}\,C + f_{kb}\,K \quad (2)$$

$$N_c = f_{ic}\,I + f_{bc}\,B + C + f_{kc}\,K \quad (3)$$

$$N_k = f_{ik}\,I + f_{bk}\,B + f_{ck}\,C + K \quad (4)$$

can be solved simultaneously by matrix algebra, using inversions to provide numerical consts W, X, Y, and Z in equations 5, 6, 7, and 8. These consts are used to solve for concn of each of these 4 nuclides in sample. Net counting rate for each nuclide is:

$$^{131}I = I = W_1N_i + W_2N_b + W_3N_c + W_4N_k \quad (5)$$

$$^{140}Ba = B = X_1N_i + X_2N_b + X_3N_c + X_4N_k \quad (6)$$

$$^{137}Cs = C = Y_1N_i + Y_2N_b + Y_3N_c + Y_4N_k \quad (7)$$

$$^{40}K = K = Z_1N_i + Z_2N_b + Z_3N_c + Z_4N_k \quad (8)$$

Calibration to derive values for consts in equations 5, 6, 7, and 8 is applicable as long as instrument alignment and mode of operation remain const and gamma-emitting nuclides are limited to the 4 elements in matrix. Long-hand inversion of 4 × 4 matrix is tedious and subject to mistakes. Use of computer is recommended to provide numerical consts for equations 5–8. Thereafter, desk calcns can det. concns of ^{131}I, ^{140}Ba, ^{137}Cs, and ^{40}K in samples in absence of computer by summing counts in each photopeak, subtracting background, and applying equations 5–8.

(c) *Iodine-131, barium-140, cesium-137, potassium-40 activities.*—From spectral gamma counts

Table 2. Collaborative results for radionuclides in milk by gamma-ray spectroscopy (pCi/L): EPA study

Sample C							
Lab.	I-131			Lab.	I-131		
1	68	71	70	22	107	99	75
2	89	90	80	23	81	90	87
3	100[a]	87[a]	—	24	79	84	82
4	88	87	88	25	82	77	80
5	74	78	70	26	86	87	88
6	74	74	71	27	88	75	85
7	86	89	81	28	74	69	74
8	82	80	86	29	87	71	75
9	87	96	114	30	72	78	61
10	88	83	99	31	83	87	86
11	92	100	90	32	84	78	79
12	73	72	69	33	104	91	102
13	79	84	89	34	77	83	75
14	75	81	75	35	78	76	78
15	76	62	74	36	80	68	83
16	83	89	79	37	69	72	77
17	87	88	83	38	78	86	90
18	76	78	80	39	79	80	81
19	76	76	82	40	88	87	90
20	72	72	72	41	86	86	89
21	76	85	80	True concn		82	

[a] Not included in statistical analysis.

Table 3. Statistical summary of 2 collaborative studies for radionuclides in milk (pCi/L)

I-131			
Sample	A	B	C
Known concentration	633	98	82
Mean result	635.3	98.9	81.6
SD within lab	29.0	6.1	5.6
between labs	30.1	8.2	7.6
Method bias[a]	+2.3 ± 10.1	+0.9 ± 2.8	−0.4 ± 3.6
Number of labs	24	24	40

Cs-137		
Sample	A	B
Known concentration	305	52
Mean result	295.2	53.3
SD within lab	11.4	4.7
between labs	13.5	4.1
Method bias[a]	−9.8 ± 4.5	+1.3 ± 1.9
Number of labs	25	25

Ba-140		
Sample	A	B
Known concentration	515	72
Mean result	522.9	76.0
SD within lab	19.5	6.5
between labs	35.8	11.2
Method bias[a]	+7.9 ± 9.8	+4.0 ± 3.4
Number of labs	24	25

[a] The range shown for each method bias is a 95% confidence limit, which is the sum of the 95% confidence limit of the mean result and the maximum error in the known concentration.

of sample, substitute net value from equations 5 thru 8 and convert net counts/min for each nuclide to pCi/L milk at time of counting:

$$^{131}I(pCi/L) = (\text{net cpm})_i/(E_i \times V)$$

$$^{140}Ba(pCi/L) = (\text{net cpm})_b/(E_b \times V)$$

$$^{137}Cs(pCi/L) = (\text{net cpm})_c/(E_c \times V)$$

$$^{40}K(pCi/L) = (\text{net cpm})_k/(E_k \times V),$$

where E_i, E_b, E_c, E_k = counting efficiency/pCi from std solns for ^{131}I, ^{140}Ba, ^{137}Cs, ^{40}K, resp., and V = sample vol., L.

Collaborative Studies

In July 1969, the Analytical Quality Control Service of the Bureau of Radiological Health sent two 1 gal. (3.78 L) milk samples, one with high and one with low concentrations of the 3 nuclides, to each of 25 laboratories (2). For each of the 6 sample–nuclide combinations, the triplicate measurements requested were supplied by all 25 laboratories in 3 of the combinations, and by all but 1 in the remaining 3 (Table 1).

In April 1978, the Environmental Monitoring Systems Laboratory-Las Vegas of the Environmental Protection Agency (EPA) prepared a milk sample with an I-131 concentration of 82 pCi/L and sent one 3.5 L aliquot to each of 60 laboratories (Table 2). Forty of the collaborators returned the 3 determinations requested, 19 did not respond, and 1 returned only a duplicate result, which was not included in the analysis (3).

Data Analysis and Discussion

An analysis of variance was performed on the data for each nuclide in each sample, 7 in all (Table 3). In the 3 sets of results for I-131, within- and between-laboratory standard deviations (SDs) are well below the actual concentrations, ranging from 5 to 8% thereof. This indicates that even the lowest of the 3 concentrations is above the detection limit of this method. The method bias is undetectable in all 3 cases, being smaller in magnitude than its uncertainty at 95% confidence. It should be noted that in the EPA study, in which 20 of the 60 laboratories failed to respond, a comparison of these results with those of the other 2 samples appears to indicate that the responding laboratories were representative, and that probably no sampling bias has been introduced by the nonrespondents.

In the 2 sets of results for Cs-137, within- and between-laboratory SDs are again small, varying from 4 to 9% of the known concentrations. The method bias is again undetectable in the low-activity sample, and although it is significant for the high-activity sample, it is only 3% of the known concentration and is smaller than either of the SDs.

Finally, the Ba-140 results show within- and between-laboratory SDs for the high-activity sample (4 and 7% of known concentration, respectively) that are similarly small, with method bias less than 2%, and not statistically significant. For the low-activity sample, within- and between-laboratory SDs are respectively 9 and 16% of known concentration, and although the method bias is again not significant, this time at less than 6% of the known concentration.

Recommendations

On the basis of the statistical analysis of results, this method is acceptable for analysis of samples containing I-131, Cs-137, and Ba-140 at, and presumably above, the levels of activity tested. It is recommended that method **48.025-48.029** be extended to include I-137 and Ba-140.

References

(1) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs. **48.025-48.029**

(2) Knowles, F. K., & Underwood, R. J. (1969) "Collaborative Study for Analysis of Radionuclides by Gamma Spectroscopy Using Simultaneous Equations," Food and Drug Administration, Bureau of Radiological Health Technical Experiment 69-MKAP-1

(3) *Environmental Radioactivity Laboratory Intercomparison Studies Program 1978-1979* (1978) Environmental Protection Agency EPA-600/4-78-032, National Technical Information Service, Springfield, VA

DRUGS IN FEEDS

Inhibition of Chlortetracycline Activity by Magnesium Ions

TOM CHIANG
Texas A&M University, Texas Agricultural Experiment Station, Agricultural Analytical Services Department, College Station, TX 77843

Up to 50% of the antimicrobial activity of chlortetracycline (CTC) can be inhibited by the presence of $MgSO_4$ in microbiological turbidimetric assays. From 50 to 200 mM $MgSO_4$ inhibited CTC activity, and the relationship between CTC inhibition and $MgSO_4$ concentration is linear. The possible mechanism of this inhibition and its potential implication are discussed.

Tetracyclines are one of the oldest families of antibiotics and yet, because of their broad antimicrobial spectrum, low toxicity, good oral absorption, and most important in the feed industry, their low cost, they are still widely used in both medicines and feeds. Chlortetracycline (CTC) was first isolated and introduced for clinical use by Duggar in 1948 (1).

The official methods for determining CTC in feeds are the AOAC plate agar-diffusion method (2) and turbidimetric method (3). In developing an automated turbidimetric assay for CTC in our laboratory, we made some interesting observations in regard to magnesium ions and the CTC assay.

It has been known for some time that tetracyclines form chelates with certain cations including magnesium (4, 5). We found that at least 50% of the antimicrobial activity of CTC can be inhibited by magnesium ions. Furthermore, if magnesium ions are added to assay broth alone, cell growth can be promoted. In view of these observations, one might expect to get different turbidimetric assay values for CTC, depending on the magnesium content in a particular feed sample. Further experiments are in progress to investigate the importance and possible solutions to these potentially interfering effects by magnesium ions.

Experimental

Apparatus and Reagents

(a) *Autoturb system.*—Elanco Products Co., Indianapolis, IN 46206. This system is used in AOAC automated turbidimetric determinations. The theory, design, construction of the Autoturb® system have been described (6). Briefly, the system consists of a dilutor, an incubation water bath, and a reader. The dilutor prepares 66- and 100-fold dilutions in pairs and delivers the diluted samples from a set of sample tubes to an array of assay tubes in a carrier. The diluent usually contains the test organism inoculated in a specified medium. These filled assay tubes in the carrier are then incubated in a water bath to allow cell growth. After the cultures reach a certain density, their concentrations are determined by the Autoturb reader which causes solution from the test tubes to flow through a fixed cell in a spectrophotometer and records percent transmittance of the flowing solutions.

(b) *Chlortetracycline (CTC).*—Prepare stock and working standard solutions as described in **42.211(a)** and **42.216(b)** (2, 3).

(c) *Dilution buffer.*—pH 4.5 phosphate buffer (**42.197(g)**) (3).

(d) *Assay broth.*—Difco antibiotic medium 3, but in 1.7× quantity as in **42.215(a)** (3).

(e) *1M $MgSO_4$ solution.*—Dissolve 169 g $MgSO_4 \cdot H_2O$ in water and dilute to 1 L.

(f) *0.1M EDTA solution.*—Dissolve 37.2 g disodium ethylenediamine tetraacetate ($C_{10}H_{14}O_8N_2 \cdot 2H_2O$) in water and dilute to 1 L; adjust pH to 7.0.

(g) *Test organism.*—*Staphylococcus aureus* ATCC 9144.

(h) *Preparation of inoculum.*—Inoculate 20 mL assay broth with 1 loop from slant culture. Incubate overnight at room temperature. Use 5–10 mL culture/L medium.

Procedure

CTC standard solutions or dilution buffer were mixed with different amounts of 1M $MgSO_4$ so that in each sample tube the final concentration of CTC was 0.8 or 0 μg/mL and of $MgSO_4$, 0, 50, 100, or 200 mM. These samples were then diluted with inoculated broth by the automated system and incubated 3–5 h at 37.5°C, and percent transmissions were recorded at 600 nm wavelength.

Received December 10, 1981. Accepted February 22, 1982.

0004-5756/82/6505-1044-04$01.00

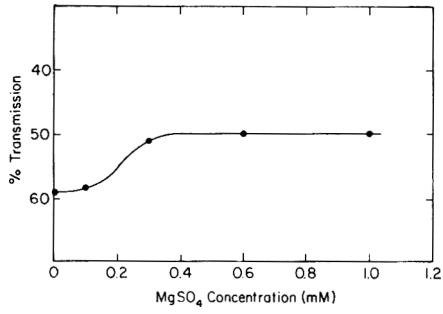


Figure 1. Percent transmission at 600 nm of post-incubation cultures responding to different concentrations of $MgSO_4$ added in growth medium (abscissa).

Table 1. Percent transmission of *Staphylococcus aureus*[a]

$MgSO_4$ concn, mM[b]	CTC concn, 0.8 μg/mL	CTC concn, 0 μg/mL
0	84.7; 84.4	59.1; 58.6
50	81.4; 81.1	58.8; 58.0
100	77.8; 77.2	58.8; 58.1
200	73.0; 72.3	58.6; 60.5
100 + 10 mM EDTA	80.7; 80.4	63.9; 62.9

[a] The assay broth was supplemented with 1 mM $MgSO_4$ (refer to text).

[b] Concentration of $MgSO_4$ in the standard CTC sample tubes or buffer sample tubes before dilution with inoculated medium (refer to procedure).

Results

Effect of Magnesium Ions on Growth of Test Organism

Figure 1 shows that if $MgSO_4$ is added to inoculated growth medium, the post-incubation percent transmission of the cultures was lower than for cultures grown without added $MgSO_4$. This indicates that additional $MgSO_4$ in the growth medium results in more cell growth. This effect on the promotion of cell growth reaches a plateau at a concentration of about 0.4 mM $MgSO_4$, as indicated by the constant percent transmission over the $MgSO_4$ concentration range from 0.4 to 1.0 mM. When $MgCl_2$ is substituted for $MgSO_4$, the same results are obtained, indicating that magnesium ions are responsible for the observed growth promotion. It is likely that magnesium ion concentration in this growth medium is not sufficient to provide optimal growth for the test organism. A determination of total magnesium concentration by atomic absorption spectroscopy showed that the value is between 0.1 and 0.2 mM for the medium used in this experiment.

Effect of Magnesium Ions on Chlortetracycline Activity

To detect the effect of magnesium on CTC activity, one has to circumvent the magnesium effect on cell growth. As shown in Figure 1, when added magnesium ion concentration in the medium reached 0.4 mM or more, no promotion of cell growth was detected. Therefore, if sufficient $MgSO_4$ is added to the medium (0.4 mM or more), cell growth should be unaffected by further changes in magnesium ion concentration. In the experiment described below, the growth medium was supplemented with 1 mM $MgSO_4$.

Table 1 shows that when different amounts of $MgSO_4$ were added to the standard CTC solution before dilution with the inoculated medium, the post-incubation percent transmission of the cultures decreased with increasing magnesium ion concentration. This indicates that the antibacterial activity of CTC is inhibited by $MgSO_4$ added to the CTC standard solution. Table 1 also shows results obtained when corresponding amounts of $MgSO_4$ were added in the absence of CTC. The resulting percent transmission values are essentially the same regardless of magnesium concentration, indicating that the extra $MgSO_4$ added to the inoculated medium (which in this case already contains extra 1 mM $MgSO_4$) did not promote cell growth. This further confirms the observation from Figure 1 that at concentrations above 0.4 mM $MgSO_4$ in the medium, the magnesium requirement for optimal growth was satisfied. When EDTA was added to the CTC standard solution plus $MgSO_4$, the inhibition of CTC activity decreased as indicated by higher percent transmission values (Table 1). This result implies that magnesium ions interact with CTC molecules to inactivate, and EDTA, probably a stronger chelating agent for magnesium, partially restores CTC activity by decreasing the effective magnesium concentration through chelation. However, because EDTA plus $MgSO_4$ alone caused some growth inhibition as also indicated in Table 1, a careful study will have to be done to further elucidate the EDTA effect quantitatively. Figure 2 shows that if the sample tube with no added $MgSO_4$ is taken as causing no inhibition, the relationship between CTC inhibition and $MgSO_4$ concentration is linear up to 50% inhibition.

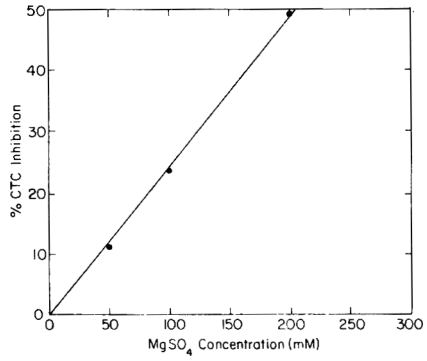


Figure 2. Inhibition of CTC activity by $MgSO_4$.

Discussion

In 1950, Gale and Paine (7) first showed, by using *Staphylococcus aureus* as test organism, that CTC was a specific inhibitor of protein synthesis. However, the molecular mechanism by which this drug inhibits bacterial growth was not understood until the detailed steps of protein synthesis were unveiled. In protein synthesis, during the growth of a polypeptide chain on a ribosome, peptide bond formation involves transfer of the nascent polypeptide from tRNA bound to one site (the "peptide donor" or "P" site) to aminoacyl tRNA bound to an adjacent site ("aminoacyl-acceptor" or "A" site). This reaction is catalyzed by an enzyme called peptidyl transferase which is an integral part of the large ribosomal subunit (8). Several groups of workers have convincingly demonstrated that the enzymatic binding of aminoacyl tRNA to the ribosome is strongly inhibited by tetracycline in vitro (9, 10) and in vivo (11). It is now widely accepted that this is the mechanism by which tetracyclines inhibit bacterial growth.

Tetracyclines are capable of chelating divalent cations such as magnesium ions (4, 5). It was suggested first by Albert (12) that chelation might play a part in their inhibitory action. Later, White and Cantor (13) used fluorescence spectroscopy to convincingly demonstrate that magnesium is involved in the binding of tetracyclines to the ribosome. In this report, we demonstrated that if CTC is treated with a high concentration of $MgSO_4$ (0.05 to 0.2M) before addition to inoculated medium, its antibacterial activity can be significantly inhibited. The logical explanation for this observation is that the CTC molecule has only limited binding sites for magnesium ions and if these sites were occupied by pre-existing magnesium ions, its binding to ribosome through magnesium chelation may be blocked. If this interpretation is correct, then one expects that EDTA would reverse the inhibitory action of magnesium ions on CTC because EDTA is a strong chelating agent for magnesium ions. Our preliminary results showed that when EDTA was added to the CTC and $MgSO_4$ mixture, the inhibitory action of magnesium ion appeared to be reduced. However, it is not clear why the corresponding control sample without CTC resulted in some inhibition of cell growth (Table 1, last line).

The magnesium effect on the promotion of cell growth reported here was unexpected. CTC turbidimetric assay is an AOAC official first action method (3). If any feed sample contains magnesium to the level that measurable growth promotion is present, the assay result could be biased low. We are investigating a means of overcoming this problem by supplementing the assay medium with extra magnesium. The addition might inhibit some CTC activity, but this is unlikely because the concentration of magnesium ions required to inhibit CTC is much higher than that present in the medium. Furthermore, even if there is minute inhibition, this should affect both the samples and the standard solutions equally.

It is important to note that the inhibition of CTC activity and the promotion of cell growth are 2 independent events even though both are exerted by magnesium. The concentration of magnesium ion needed for inhibition of CTC activity is more than 2 orders of magnitude higher than the concentration needed for promotion of cell growth (compare Figures 1 and 2). However, in either case, the result is to cause low bias in feed sample analysis.

Acknowledgment

The author thanks Alan R. Hanks for his helpful discussion and criticism of the manuscript, and Sheila Angell and Tammy Burgess for determining the magnesium content in the assay broth by atomic absorption spectroscopy.

References

(1) Duggar, B. M. (1948) *Ann. N. Y. Acad. Sci.* **51**, 177-183
(2) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **42.211-42.213**
(3) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **42.215-42.220**
(4) Kohn, K. W. (1961) *Anal. Chem.* **33**, 862-866
(5) Grassi, G. G., & Sabath, L. D. (1981) *New Trends in Antibiotics: Research and Therapy*, Elsevier/North-Holland Biochemical Press, Amsterdam, Netherlands, pp. 1-44

(6) Kuzel, N. R. & Kavanagh, F. W. (1971) *J. Pharm. Sci.* **60,** 764–767; 767–773
(7) Gale, E. F., & Paine, T. F. (1950) *Biochem. J.* **47,** XXVI–XXVII
(8) Traut, R. R., & Monro, R. E. (1964) *J. Mol. Biol.* **10,** 63–72
(9) Lucas-Lenard, J., & Haenni, A. L. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **59,** 554–560
(10) Ravel, J. M., Shorey, R. L., Garner, C. W., Dawkins, R. C., & Shive, W. (1967) *Cold Spring Harbor Symp. Quant. Biol.* **32,** 543–545
(11) Cundliffe, E. (1972) *Molecular Mechanisms of Antibiotic Action on Protein Biosynthesis and Membranes,* Elsevier Scientific Publishing Co., Amsterdam, The Netherlands
(12) Albert, A. (1958) *Symp. Soc. Gen. Microbiol.* **8,** 112–138
(13) White, J. P., & Cantor, C. R. (1971) *J. Mol. Biol.* **58,** 397–400

Rapid Gas-Liquid Chromatographic Method for Determination of Sulfamethazine in Swine Feed

ROBERT K. MUNNS and JOSE E. ROYBAL
Food and Drug Administration, Animal Drug Research Center, 500 U.S. Customhouse, Denver, CO 80202

A gas-liquid chromatographic method is described for the quantitative determination of trace amounts of sulfamethazine in swine feed. Sulfamethazine is extracted in ammoniated acetone and isolated from other extractants on a Sephadex LH-20 column. The eluate is methylated with diazomethane and evaporated to dryness. The residue is dissolved in a solvent containing an internal standard of methyl sulfasymazine before being injected onto an OV-25 GLC column. An estimation of precision was established by assaying 10 sets of swine feed fortified with 0.5, 1, 2, and 5 ppm SMZ. Mean recoveries were 96.0, 94.3, 93.5, and 94.0%, respectively, with an average coefficient of variation of 3.07%. The critical steps and ruggedness of the method were also determined.

Sulfamethazine is traditionally determined by the Bratton-Marshall colorimetric methods (1, 2), which lack both specificity and sensitivity. High pressure liquid chromatography (HPLC) methods, to a large extent, overcome these deficiencies. Quantitative methods using HPLC specify a variety of column conditions for various products. For example, an ion exchange column was used for sulfaquinoxaline in medicated feed (3) and a bonded aminophase column was used to chromatograph sulfonamides and their N^4-acetylated metabolites in urine samples (4). Johnson et al. (5) used a bonded C_{18}-phase column to assay bovine tissue for sulfonamides. Mixtures of sulfa drugs were also separated and determined by HPLC by aqueous-buffered ion-pair chromatography (6) and with a silica gel column (7). But the versatility of HPLC residue analysis is limited, because the sensitivity of any method depends on the cleanup of large sample portions.

A gas chromatograph equipped with an electron capture (EC) detector can detect residual amounts of sulfonamides, and smaller sample portions can be utilized for the cleanup procedure. Gas-liquid chromatography (GLC) methods (8) have been reported in the literature, including a procedure by Goodspeed et al. (9) in which the sulfonamides in tissue are determined after methylation with diazomethane followed by formation of a second derivative with pentafluoropropionic anhydride. They determined the double derivative on an OV-17 column with a ^{63}Ni detector. Crisp (10) reported a method for determining sulfaquinoxaline in eggs and poultry meat by first hydrolyzing it to 2-aminoquinoxaline and then derivatizing with trifluoroacetic anhydride; the derivative is separated on a neopentylglycol column and detected with a tritium EC detector. The preparation of perfluoro derivatives of some sulfonamides and column conditions are described by Gyllenhaal and Ehrsson (11).

GLC generally requires a more rigorous cleanup procedure than either colorimetric or HPLC methods. King et al. (12) discussed a cleanup procedure for diethylstilbestrol in animal feed which specifies a Sephadex LH-20 column. We find the Sephadex cleanup effective for other animal feeds as well.

This study was undertaken to provide a method that minimizes animal feed interferences sufficiently so that sulfamethazine can be determined in trace amounts by GLC-EC. A thin layer chromatography (TLC) test to confirm the presence of sulfamethazine is also presented.

METHOD

Reagents

(a) *Solvents.*—Methanol, ethyl acetate, hexane, toluene, acetone, chloroform, *n*-butanol; all distilled-in-glass (Burdick and Jackson, Inc., Muskegon, MI 49442); anhydrous ethyl ether (J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

(b) *Sephadex LH-20.*—25–100 μm size (Pharmacia Fine Chemicals Inc., Piscataway, NJ 08854).

(c) *Carbitol.*—2-(2-Ethoxy) ethanol (Eastman Organic Chemicals, Rochester, NY 14650).

(d) *Standards.*—Sulfamethazine reference standard, USP. Prepare 100 μg/mL stock solution in methanol and use it to prepare 1.0 μg/mL working standard in methanol.

(e) *Diazald.*—*N* - Methyl - *N* - nitroso - *p* - toluenesulfonamide (Eastman Organic Chemicals, Rochester, NY 14650).

(f) *Diazomethane.*—Weigh 600 mg diazald into

Received January 11, 1982. Accepted February 18, 1982.

0004-5756/82/6505-1048-06$01.00

reaction tube with bubbler. Add 2 mL anhydrous ether and carbitol, and mix. Insert delivery tube of diazomethane generator into test tube containing 20 mL anhydrous ether and place test tube in ice water bath. Add 2 mL 60% KOH to reaction tube and quickly connect bubbler. Very slowly sweep reaction tube with nitrogen until yellow color of diazomethane is carried over to ether. Continue nitrogen flow until no further increase in intensity is noted (ca 5 min). Stopper test tube with cork stopper and store in freezer. Use within 3 days of preparation.

(Caution: Work with diazomethane in a fume hood! See Sax, N.I. (1975) *Dangerous Properties of Industrial Materials*, 4th ed., Van Nostrand Reinhold Co., New York, NY, p. 610.)

(g) *Internal standard (Istd).*—Sulfasymazine (Lederle Laboratories Div., American Cyanamid Co., Pearl River, NY). *Stock solution.*—100 μg/mL in methanol. *Working standard.*—10 μg/mL in methanol.

(h) *Diluting solvent with internal standard.*—Transfer 2.0 mL working Istd into 200 mL volumetric flask. Add 0.5 mL diazomethane solution. Stopper, and let stand 10 min. Evaporate at 40°C to dryness with nitrogen. Dilute to volume with ethyl acetate–hexane (3 + 7).

Apparatus

(a) *Gas chromatograph.*—Tracor Model 220 equipped with ^{63}Ni electron capture detector. Column: 1.8 m × 4 mm id packed with 100–120 mesh Supelcoport coated with 3% OV-25. Operating conditions: detector 325°C, column 270°C, inlet 290°C; argon–methane (90 + 10) carrier gas flow rate 60 mL/min. Condition column 16 h at 300°C with nitrogen carrier gas with flow rate between 10 and 20 mL/min.

A 3 μL injection of derivatized SMZ standard at 0.2 μg/mL with Istd should give at least 50% FSD at 16×10^{-2} amp/mV with retention time of 4 min for SMZ. Column should completely resolve standard and Istd peaks.

(b) *Rotary evaporator.*—Buchi Rotavapor (Brinkmann Instruments, Westbury, NY 11590).

(c) *Evaporator.*—Meyer Nitrogen-EVAP Model 112 (Organomation Associates Inc., Shrewsbury, MA 01545).

(d) *Diazomethane generator.*—Glass bubbler was constructed with side arm, and ⅋ 24/40 clear seal joint was connected to 20 mL reactor tube. (See Figure 1.) Diazomethane was collected in test tube containing chilled ethyl ether.

(e) *Chromatographic tubes.*—22 mm id × 300 mm (K-420550-233, Kontes Glass Co., Vineland, NJ 08368).

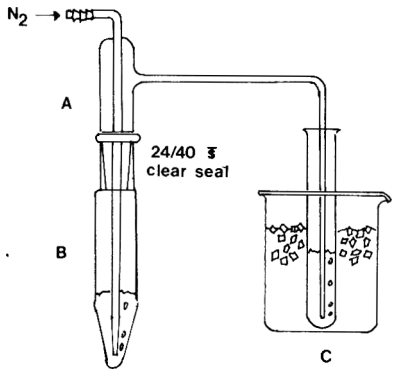


Figure 1. Diazomethane generator: A, bubbler tube with ca 1 mm id orifices; B, reaction tube with ca 20 mL volume; C, test tube receiver with ice water bath.

(f) *Centrifuge bottle.*—250 mL with stopper (K-322000-21 and K-850400 ⅋ 29/26, Kontes Glass Co.).

(g) *Distilling flask.*—100 mL pear-shaped flask with 24/40 ⅋ joint (K-608700, Kontes Glass Co.).

(h) *Thin layer chromatographic (TLC) plates.*—20 × 20 cm precoated with 0.25 mm layer of silica gel 60 (No. 5765, E Merck, Darmstadt, Germany).

Preparation and Evaluation of Sephadex Column

Add 10.0 g dry Sephadex to chromatographic tube containing 60 mL methanol, stopper column, and mix. Wash column wall with additional methanol. After 1 h, drain column and wash Sephadex with four 15 mL portions of methanol, followed by four 15 mL portions of methanol-toluene (1 + 3), retaining about 5 cm solvent above bed. Let column equilibrate 1 h before use. If Sephadex is not uniform, stopper column, mix by inversion, and let bed resettle.

Evaporate to dryness an aliquot of standard equivalent to ca 5 μg SMZ. Transfer to Sephadex column as described in cleanup procedure. Continue to elute until sixteen 10 mL fractions are collected. Add 0.5 mL diazomethane solution to each fraction, stopper, and let stand 10 min. (Caution: Handle in hood.) Evaporate to dryness at 70°C with nitrogen. Initially, dissolve each fraction in 1 mL diluting solvent with Istd and inject into GLC column. Use same diluting solvent to make further dilutions. Calculate amount of SMZ in each fraction by comparison to standard SMZ described in derivatization procedure.

Plot volume of eluate vs concentration of each fraction. If SMZ first appears in fifth fraction, use 40 mL forerun volume. If SMZ appears in fourth fraction but at less than 5% of total, use 35 mL forerun. More than 95% of the SMZ should be collected before tenth fraction. If amount of SMZ found in fractions is outside these limits, adjust ratio of methanol in eluting solvent to achieve proper elution profile. Additional methanol will decrease retention volume of sulfonamides.

Regenerate column only if background of chromatogram appears to increase. To regenerate Sephadex column, pass four 15 mL volumes of methanol through column, followed by four 15 mL portions of methanol-toluene (1 + 3). Let each portion enter bed before adding next. Let equilibrate at least 1 h before use.

Extraction

Weigh 50.0 g well mixed feed previously ground to pass 2 mm screen into 250 mL centrifuge bottle. Add 100 mL 2% NH_4OH-acetone and shake vigorously 15 min on mechanical shaker. Centrifuge 10 min at 2000 rpm or until supernate is clear. Decant into flask, and stopper. Store extracts in refrigerator.

Cleanup

Pipet 4.0 mL clear supernate into 15 mL centrifuge tube. Evaporate *just* to dryness at 40°C with nitrogen. Add 2 mL methanol-toluene (1 + 3) eluting solvent. Caution: Discontinue evaporation at first indication of dryness; do not let sample stand in eluting solvent. Proceed to Sephadex column as soon as possible.

Drain solvent in column to level of Sephadex bed and place 50 mL graduated cylinder under column. Transfer residue to column with four 2 mL additional portions of methanol-toluene (1 + 3). Let each addition enter bed before adding next portion, using care not to disturb column bed. Add solvent to maintain 1–20 cm liquid above bed. Collect first 40 mL forerun eluate or modify as necessary (see *Preparation and Evaluation of Sephadex Column*). Wash tip of column with solvent and discard wash. Collect the next 70 mL eluate in 100 mL pear-shaped flask. Wash tip with solvent.

Derivatization

Add 0.5 mL diazomethane to eluate, mix, and stopper. Let stand 10 min and rotary-evaporate at 60–70°C to dryness. Cool flask and add 5.0 mL diluting solvent with Istd. Stopper, and mix.

Pipet 2.0 mL working SMZ standard into 100 mL pear-shaped flask. Add 70 mL methanol-toluene (1 + 3), derivatize as above, evaporate, and dilute to 10 mL with diluting solvent with Istd. Inject 3 μL into gas chromatograph. Bracket samples with standard SMZ injections.

$$\text{ppm SMZ} = [\text{PH SMZ (sample)}/\text{PH SMZ (std)}] \times [\text{PH Istd (std)}/\text{PH Istd (sample)}] \times \text{concn SMZ std } (\mu\text{g/mL, final column}) \times (5.0\text{ mL}/4.0\text{ mL}) \times (100\text{ mL}/50.0\text{ g})$$

TLC Identification of Sulfamethazine

Treat a second 4.0 mL aliquot of sample extract as in cleanup step except do not derivatize sample. Evaporate to dryness in rotary evaporator at 60–70°C.

Dilute samples to appropriate volume with acetone-toluene (1 + 1) and spot ca 0.5 μg of each sulfonamide on 2 TLC plates. Develop one plate in toluene-acetonitrile (1 + 1) and the other plate in chloroform-methanol-*n*-butanol-ammonium hydroxide (75 + 15 + 9 + 1).

After development, transfer TLC plate to empty development tank to diazotize SMZ. Add 1 g sodium nitrite to 50 mL beaker, and place beaker into tank. Pipet ca 5 mL concentrated HCl into beaker and quickly cover tank. Remove TLC plate after 2 min and spray with 0.1% *N*-1-naphthylethylenediamine dihydrochloride in methanol. Rose-colored spots denote compounds having arylamine group. (Caution: HNO_2 fumes are toxic; work in hood.)

Results and Discussion

The following procedure of preparing feed samples fortified with SMZ was used to test the precision and ruggedness of the proposed method. A locally produced commercial finishing hog feed was ground to pass a 0.5 mm screen. Two hundred mg sulfamethazine USP was added to about 200 g of this feed in a quart glass jar. The mixture was tumbled for 16 h to ensure a thoroughly homogeneous mixture. Ten assay portions were taken at 1 min intervals after mixing, and the concentration of SMZ was determined according to the official AOAC method (2): 991.2, 975.8, 986.1, 975.8, 991.2, 970.7, 988.7, 988.7, 968.1, 973.3, mean 980.96, SD 9.059, CV 0.923%. The data show that a reasonable degree of uniformity was achieved.

This feed mixture was designated as the stock standard from which the working standards were made. Additional portions of the commercial feed were finely ground and mixed with portions of the standard stock to yield concen-

Table 1. Determination of precision of proposed GLC method for determining sulfamethazine

	Spike level, ppm							
	0.499		0.982		1.962		4.905	
Assay No.	Rec.	%	Rec.	%	Rec.	%	Rec.	%
1	0.465	93.2	0.906	92.3	1.796	91.5	4.267	87.0
2	0.499	100.0	0.899	91.5	1.824	93.0	4.808	98.0
3	0.469	94.0	0.925	94.2	1.904	97.0	4.760	97.0
4	0.463	92.8	0.938	95.5	1.894	96.5	4.828	98.4
5	0.458	91.8	0.935	95.2	1.794	91.4	4.636	94.5
6	0.494	99.0	0.928	94.5	1.792	91.3	4.617	94.1
7	0.479	96.0	0.933	95.0	1.835	93.5	4.626	94.3
8	0.455	91.2	0.937	95.4	1.821	92.8	4.441	90.5
9	0.520	104.2	0.932	94.9	1.869	95.3	4.579	93.4
10	0.488	97.8	0.922	93.9	1.789	91.2	4.530	92.4
Mean	0.479	96.0	0.926	94.3	1.832	93.4	4.609	94.0
SD	0.0210		0.0132		0.0432		0.171	
CV, %	4.38		1.43		2.36		3.71	

trations of about 1.25, 2.5, 25, 50, 100, and 250 ppm SMZ. When 1.000 ± 0.002 g diluted spike was mixed with 49.00 ± 0.01 g, the final spike levels were 0.025, 0.05, 0.5, 1, 2, and 5 ppm, respectively.

The precision of the proposed method was tested (Table 1) with the 4 highest spike levels for reasons that will be discussed later. Again, 10 sets of assays were made at different intervals, and the results show average recoveries of 96.0, 94.4, 93.5, and 94.0% for the 0.5, 1, 3, and 5 ppm levels, respectively. Precision was achieved with the use of an internal standard, which gave coefficients of variation of 4.38, 1.43, 2.36, and 3.71%, respectively, rather than the 5.82% coefficient of variation obtained without the use of an internal standard.

To establish the utility of the method, 4 different types of swine feeds were assayed. These feeds, vitamin- and mineral-enriched, represented the largest variety of plant materials available locally at the time. These samples were fortified with the 4 spike levels in the manner described earlier. The results are given in Table 2.

The assay results of samples spiked at less than 0.5 ppm varied between assays and tended to be higher than 100% recovery.

To obtain SMZ at 0.05 and 0.025 ppm, 2 approaches were taken. First, the proposed method was used on an equivalent of 2 g feed for cleanup, except for the final concentration, which was diluted to represent 1 g/mL. The second approach was to carry an equivalent of 10

Table 2. Assay results for sulfamethazine in 4 different types of spiked commercial swine feeds by proposed GLC method

	Spike Level, ppm							
	0.499		0.982		1.962		4.905	
Feed	Rec.	%	Rec.	%	Rec.	%	Rec.	%
Co-op Hog Finisher-14 Complete (A)	0.404	81.0	0.896	91.2	1.709	87.1	4.508	91.9
Co-op Sow Lactation	0.471	94.4	0.923	94.0	1.896	96.6	4.563	93.0
Purina Complete Sow Chow w/o	0.467	93.6	0.910	92.7	1.764	89.9	4.428	90.3
Co-op Swine[a] Builder (A)	0.422	84.6	0.843	85.8	1.796	91.5	4.217	86.0

[a] Sample contained large amounts of alfalfa.

Table 3. Effect on SMZ recovery of prolonged evaporation treatment after solvent removal

Approx. time in dry state, min	Recovery, %			
	0.499 ppm	0.982 ppm	1.962 ppm	4.905 ppm
0–1	100.0	91.5	92.9	98.0
20	75.8	73.2	72.8	75.1

g feed through the cleanup. This solution was also diluted to 1 g/mL final concentration. The 10 g cleanup resulted in somewhat lower recoveries and the interferences were appreciably higher than with 2 g feed. The use of the Sephadex column is a rapid cleanup procedure; however, the purification is more complete with a smaller amount of material. The practical limits of determining SMZ in feed is about 0.25 ppm, unless additional cleanup steps are used or included.

In testing the proposed method for ruggedness, certain steps were critical. The length of time SMZ was exposed to feed material in solution tended to affect the recovery of SMZ. For example, a 10 min extraction time gave higher recoveries than a 30 min extraction. Table 3 shows a 25% loss in recovery after a 30 min delay in removing samples from drying at 40°C under nitrogen. There is some loss in recovery when the residue remains dissolved in the eluting solution too long. It is recommended that the extraction be carried through the cleanup step as quickly as possible. Sulfamethazine is stable in 2% NH_4OH–acetone for a week if kept refrigerated. Other stability factors are reported in Table 4.

Changes in the Sephadex elution profile column have not occurred. The elution profile of SMZ in a feed matrix from a Sephadex column when the column was first prepared appears to be the same as an SMZ profile after the column was used more than 50 times (Figure 2). Regenerating the Sephadex column is necessary only if excessive amounts of feed material were passed through the column. The performance of the Sephadex column has been consistent throughout this study.

The OV-25 liquid phase packing used for GLC was chosen originally because it would resolve an interference peak that had a retention time similar to sulfathiazole. This phase, as it turned out, gave a baseline separation of the sulfasymazine and SMZ peaks. A typical chromatogram is shown in Figure 3. The precision in recovery of SMZ is improved because any instability in detector response resulting from background will affect the peak heights of SMZ and the internal standard equally; therefore, the ratio of SMZ/internal standard remains constant regardless of matrix effect. Since the proposed method does not have a rigorous cleanup step, the background will be different depending on the sample dilution. Variations in peak heights of sulfa compounds of as much as 10% are not unusual in analyses of 4 samples (9 injections). After 20 or more sample analyses, peak broadening may occur; it can be corrected by replacing the glass wool at the head of the GC column.

Thin layer chromatography.—The solvent systems described in the proposed method separated the principal sulfa compounds that are

Table 4. Stability of SMZ under various conditions

Matrix	Period tested[a]	Comment
SMZ std in methanol	6 months	stored in actinic glass at room temp.
SMZ mixed with feed	4½ months	glass jars at room temp.
SMZ in 2% NH_4OH–acetone ext	1 week	refrig. at 4°C
SMZ in methanol–toluene (1 + 3) eluting solv.[b]	3 days	after Sephadex cleanup; room temp.
Methylated SMZ + sulfasymazine (Istd)[b]	1 week	room temp.
SMZ during rotary evapn of eluting solv.[b]	1 h	tested up to 70°C

[a] Probably stable longer than period of test.
[b] In the presence of feed material.

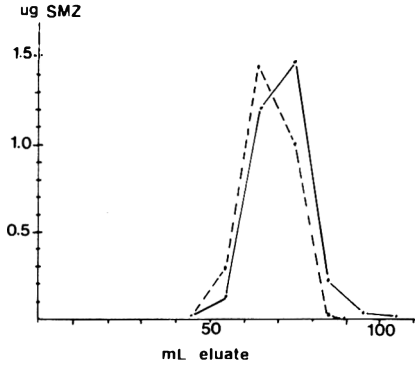


Figure 2. Typical elution profile of spiked feed after preparation of Sephadex column (—). Elution profile of Sephadex column after ca 50 samples (---).

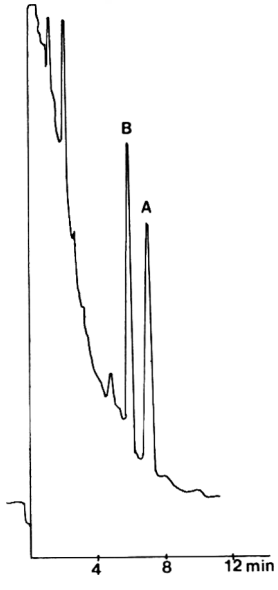


Figure 3. Gas chromatogram of extract from hog feed: A, sulfamethazine at 0.5 ppm; B, sulfasymazine internal standard.

present in feed fed to swine. The R_f values for SMZ were 0.5 and 0.4 for the first and second solvent systems, respectively. The methylated solution from the assay has also been used to identify SMZ. However, the solvent systems were altered, which gave a lower R_f value; methanol–chloroform (1 + 9) or *t*-butanol–chloroform (15 + 85) were used with good results.

Acknowledgments

The author is indebted to Lederle Laboratories for supplying the sulfasymazine standard.

References

(1) Bratton, A. C., & Marshall, E. K. (1939) *J. Biol. Chem.* **128,** 537–550
(2) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, sec. **42.172**
(3) Cox, G. B., & Sugden, K. (1977) *Analyst* **102,** 29–34
(4) Sharma, J. P., Perkins, E. G., & Bevill, R. F. (1976) *J. Pharm. Sci.* **65,** 1606–1608
(5) Johnson, K. L., Jeter, D. T., & Claiborne, R. C. (1975) *J. Pharm. Sci.* **64,** 1657–1660
(6) Rader, B. R. (1973) *J. Pharm. Sci.* **62,** 1148–1150
(7) Cobb, P. H., Hill, G. T. (1976) *J. Chromatogr.* **123,** 444–447
(8) Malanoski, A. J., Barnes, C. J., & Fazio, T. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1386–1391
(9) Goodspeed, D. P., Simpson, R. M., Ashworth, R. B., Shafer, J. W., & Cook. H. R. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1050–1053
(10) Crisp, S. (1971) *Analyst* **96,** 671–674
(11) Gyllenhaal, O., & Ehrsson, H. (1975) *J. Chromatogr.* **107,** 327–333
(12) King, J. R., Nony, C. R., & Bowman, M. C. (1977) *J. Chromatogr. Sci.* **15,** 14–21

DRUGS

Drug Quality Assessment Methods. I. Gas-Liquid Chromatographic Assay and Identification of Seven Barbiturates

DONALD B. BLACK, HALINA KOLASINSKI, EDWARD G. LOVERING,[1] and JAMES R. WATSON

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

A gas-liquid chromatographic (GLC) procedure has been developed for the assay and identification of amobarbital, butabarbital, heptabarbital, mephobarbital, pentobarbital, phenobarbital, and secobarbital in single component capsule, elixir, injectable, suppository, and tablet formulations. After extraction into chloroform from an acidified aqueous mixture of the product, the drug is eluted isothermally from a methylphenylsilicone GLC column at 210 or 240°C and quantitated relative to thiamylal internal standard. Results were in good agreement with those obtained using pharmacopeial assay methods. The method is suitable for the rapid assessment of commercial formulations.

Monographs for drug formulations in the *U.S. Pharmacopeia* (1) and *British Pharmacopoeia* (2) typically contain requirements for drug identity and content. Depending on the nature of the drug, the dosage form, and its strength, they may also contain requirements for content uniformity, for chemical impurities related to the drug, and for other tests which reflect drug product quality. In many cases, the methods for each test or assay differ from each other and from the same test or assay in chemically related drugs.

In large scale programs where the pharmaceutical quality of several thousand drug products is evaluated by testing to pharmacopeial or similar standards, it would be advantageous if methods were available which provided data to assess more than one pharmacopeial requirement, and which could be used for groups of related drugs rather than single drugs. Modern chromatographic techniques offer some of these advantages. For example, identity can be assessed by retention time, drug content can be determined by the mean of content uniformity assays, and drug-related impurities can be resolved and quantitated by an appropriate chromatographic system. Such a method for 3 benzodiazepine drugs was recently developed in this laboratory (3).

A gas-liquid chromatographic method for content uniformity, assay, and identity of barbiturate formulations is described in this paper. Experimental work was confined to barbiturates available in Canada, namely, amobarbital, butabarbital, heptabarbital, mephobarbital, pentobarbital, phenobarbital, and secobarbital, but it is expected that the method could be used for other barbiturates. Current USP methods (1) for the assay and content uniformity of barbiturate formulations include gravimetric, titrimetric, ultraviolet, and gas chromatographic procedures. Column chromatographic procedures for several barbiturate formulations have been developed (4–6), and the current literature contains numerous reports of gas (7–15) and liquid (16, 17) chromatographic methods for barbiturates in dosage forms and biological fluids. The USP gas chromatographic system for barbiturates (1) was not used as the basis for this work because systems exhibiting superior chromatographic characteristics were available at the time.

Experimental

Apparatus and Reagents

(a) *Gas chromatograph.*—Bendix Model 2500, equipped with Hewlett-Packard Model 3380A reporting integrator, flame ionization detector, and 1.8 m × 4 mm id glass column packed with commercial acid-deactivated methylphenylsilicone (Supelco Inc.) on acid-washed, dimethylchlorosilane-treated diatomite support (3% SP 2250 DA on 100–120 mesh Supelcoport).

(b) *Drugs.*—Raw materials for use as standards were obtained as follows: thiamylal (internal standard), Parke-Davis and Co., Brockville, Ontario; amobarbital, May and Baker, Toronto, Ontario; butabarbital, Geigy, Montreal, Quebec; heptabarbital, May and Baker, Toronto, Ontario; mephobarbital, extracted from Mebaral® tablets, Winthrop, Aurora, Ontario; pentobarbital, ex-

[1] Direct correspondence to this author.

Received September 3, 1981. Accepted February 10, 1982.

0004-5756/82/6505-1054–05$01.00

Table 1. Suitability and extraction data for GLC of barbiturates

Barbiturate	Tailing factor	Resolution	Unrecovered drug, %
Amobarbital	1.8	7.2	0.3
Butabarbital	1.6	7.9	0.3
Heptabarbital	1.5	7.5	0.2
Mephobarbital	1.4	2.5	0.2
Pentobarbital	1.8	6.2	0.4
Phenobarbital	1.6	5.0	0.4
Secobarbital	1.5	4.1	0.2

tracted from Nembutal® capsules, Abbott, Montreal, Quebec; phenobarbital, USP Reference Standard; secobarbital extracted from Secogen® capsules, Paul Maney, Toronto, Ontario. The identity of all barbiturate samples used as standards was confirmed by comparison of proton magnetic resonance spectra with authenticated barbiturate spectra. Barbiturates extracted from formulated products were assayed by ultraviolet absorption against the USP Reference Standard (mephobarbital and pentobarbital) or other standard (May and Baker secobarbital).

(c) *Solutions.*—(1) *Internal standard solution.*—2.0 mg thiamylal/mL $CHCl_3$. (2) *Standard barbiturate preparations.*—Prepared to contain ca 1 mg barbiturate/mL by mixing equal volumes of internal standard solution and $CHCl_3$ solution (2 mg/mL) of USP Reference Standard barbiturate or other acceptable standard.

System Suitability

Five aliquots of standard barbiturate preparation were injected and peak responses were recorded. Relative standard deviation for R_s, area ratio of barbiturate to thiamylal peaks, should not be >1.5. Resolution and tailing were assessed in terms of criteria given in USP XX, Section 621.

Procedure

(a) *Content uniformity preparation (capsules and tablets).*—Single tablets, or the entire contents of single capsules, were placed into each of 10 glass tubes of suitable size fitted with Teflon-lined screw-caps. Each tablet was crushed with a glass rod, and 2 mL 2.4N HCl and sufficient $CHCl_3$ to

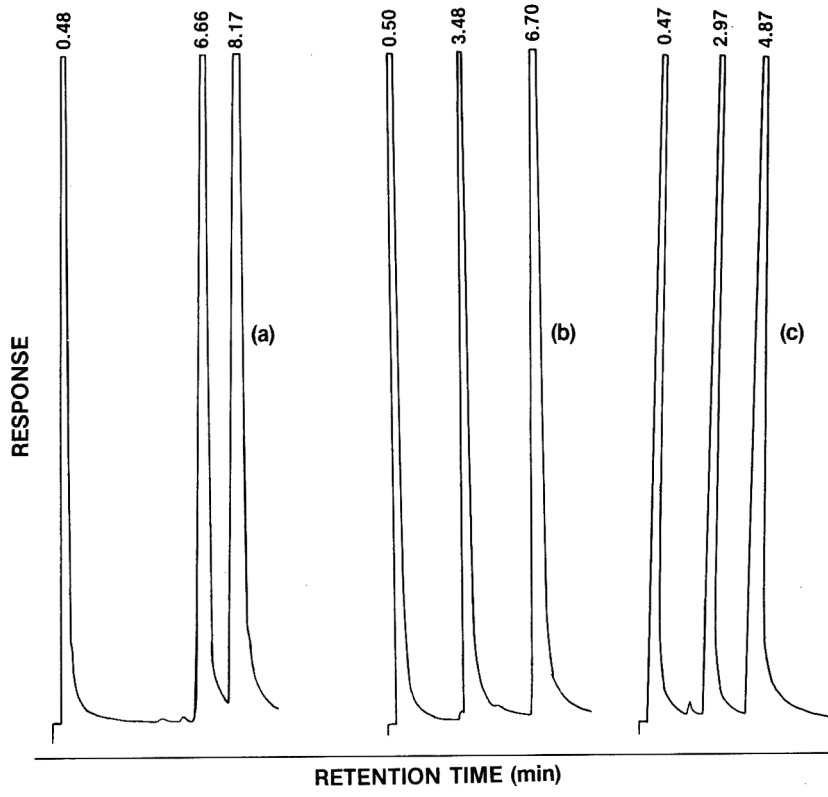


Figure 1. Typical chromatograms: (a) mephobarbital, 8.17 min; thiamylal, 6.66 min. (b) amobarbital, 3.48 min; thiamylal, 6.70 min. (c) phenobarbital (240°C), 4.87 min; thiamylal, 2.97 min.

Table 2. Retention time and response characteristics for GLC of barbiturates

Drug	Concentration,[a] mg/mL	Relative retention time[b]	Concentration–area response ratio[c]
Amobarbital	0.708	0.516	0.967
	1.062	0.516	0.948
	1.416	0.514	0.940
Butabarbital	0.422	0.471	1.230
	0.845	0.470	1.207
	1.265	0.470	1.204
Heptabarbital[d]	0.492	1.89	1.025
	0.988	1.90	1.006
	1.580	1.91	1.006
Mephobarbital	0.510	1.21	1.043
	1.020	1.21	1.012
	1.530	1.21	1.009
Pentobarbital	0.524	0.584	1.101
	1.047	0.581	1.087
	1.570	0.579	1.096
Phenobarbital[d]	0.740	1.57	0.948
	0.990	1.56	0.921
	1.350	1.57	0.912
Secobarbital	0.560	0.684	1.094
	1.120	0.678	1.084
	1.680	0.680	1.089

[a] The procedure calls for a concentration of 1 mg/mL in the standard and content uniformity preparations; 4 μL is injected.
[b] Relative to thiamylal, the internal standard. For thiamylal in our system, retention times were 6.7 and 2.9 min at 210 and 240°C, respectively.
[c] Concentration in mg/mL. Area response is ratio of areas of drug to internal standard peaks.
[d] Column temperature 240°C.

give a final concentration ca 2 mg/mL were added. Tubes were shaken and centrifuged, and the aqueous layer was discarded.

(b) *Assay preparation (elixirs and injectables).*—An amount of elixir or injectable equivalent to ca 50 mg barbiturate was transferred accurately to a separatory funnel, and 2 mL 2.4N HCl was added. The solution was mixed and extracted with two 10 mL portions of $CHCl_3$. The $CHCl_3$ extracts were collected in a 25 mL volumetric flask and diluted to volume with $CHCl_3$.

(c) *Assay preparation (suppositories).*—Ten suppositories were accurately weighed, transferred to a 250 mL separatory funnel, and shaken with 100 mL 0.1N NaOH and 50 mL $CHCl_3$ until completely dissolved. The $CHCl_3$ layer was discarded. The aqueous layer was quantitatively transferred to a 250 mL volumetric flask and diluted to volume with 0.1N NaOH. A volume of solution equivalent to ca 50 mg barbiturate sodium was pipetted into a clean separatory funnel, acidified with 2.4N HCl, and extracted with two 10 mL portions of $CHCl_3$. $CHCl_3$ extracts were combined and diluted to 25 mL.

Analysis

One mL of the assay or each of the content uniformity preparations was mixed with 1 mL Internal Standard Solution, and equal volumes (ca 4 μL) of this solution and the Standard Barbiturate Preparation were injected into the chromatograph. The chromatograms were recorded and the responses of the barbiturate and internal standard peaks were measured. For capsules and tablets, the assay value can be determined from the mean of the 10 single tablet or capsule assays required for content uniformity, or a composite of 10 or 20 dosage units can be made and an amount equivalent to 50 mg of drug can be assayed. For elixirs, injectables, and suppositories, the assay value is calculated relative to the standard barbiturate preparation, making allowances for the appropriate conversion and dilution factors in the computation of results. Conversion factors, sodium salt/free acid: amobarbital, 1.0972; butabarbital, 1.1036; pentobarbital, 1.0972; phenobarbital, 1.0946; secobarbital, 1.0922.

Results and Discussion

Under the conditions chosen for this method, the chromatographic peaks of the 7 barbiturates examined were sharp and well resolved from the solvent front. Typical values for resolution of the drug from the internal standard and the tailing factor are given in Table 1, and typical

Table 3. Comparison of proposed GLC assay and USP assay for different formulations of 7 barbiturates

Drug	Dose form and strength	Lot No.	GLC assay, %		USP assay,[b] %
			Mean ± CV[a]	Range	
Amobarbital	tabs, 30 mg	A	98.5 ± 1.8	95.3–101.3	97.2[c]
Amobarbital sodium	caps, 200 mg	B	100.1 ± 3.1	93.8–106.9	99.8
Butabarbital sodium	elixir, 6 mg/mL	C	96.1[b]		96.8
Heptabarbital	tabs, 200 mg	D	101.3 ± 2.2[d]	98.4–103.6	—[e]
Mephobarbital	tabs, 30 mg	E	102.5 ± 2.9	96.4–105.2	101.4[c]
	tabs, 100 mg	F	102.2 ± 0.8	101.2–103.7	102.2[c]
Pentobarbital sodium	caps, 100 mg	G	98.0 ± 1.4	96.4–100.1	96.3
		H	96.5 ± 2.9	92.1–102.2	97.4
		I	97.0 ± 1.5[f]	94.5–98.4	99.7
	supp., 50 mg	J	96.2[b]		—[e]
Phenobarbital	tabs, 15 mg	K	100.4 ± 1.4	98.8–102.9	101.8
	tabs, 30 mg	L	98.3 ± 2.7	92.7–103.1	100.8
	tabs, 100 mg	M	100.3 ± 0.9	99.1–102.1	99.5
	elixir, 4 mg/mL	N	96.3[b]		97.7
Phenobarbital sodium	inject., 120 mg/mL	O	94.2[b]		94.2
Secobarbital sodium	caps, 50 mg	P	99.0 ± 5.0	90.0–107.3	98.6
	caps, 100 mg	Q	99.9 ± 1.9	97.5–103.0	98.7
		R	100.2 ± 3.5	95.5–106.6	98.9
		S	98.5 ± 5.5	91.2–116.7	97.4

[a] Mean of 10 tablet or capsule assays, plus or minus the coefficient of variation.
[b] Duplicate determinations.
[c] NF XIV assay procedure.
[d] Mean of 5 tablets.
[e] Not in USP XIX or NF XIV.
[f] An unidentified peak (relative retention time 0.53), equivalent in area to 3% was observed in this product.

chromatograms are given in Figure 1. Relative retention times were reproducible (Table 2); the coefficient of variation for each drug was 0.5% or less. The amount of drug recovered by an extraction additional to that called for in the method is given in Table 1. In no case was more than 0.5% of the drug lost; this is acceptable for the purpose of this method. In this work, the identity of the drug in the product was considered to be corroborated when the relative retention time of the drug peak in the assay chromatogram was within 0.5% of the relative retention time of the barbiturate in the standard preparation chromatogram. The method requires that concentration of barbiturate in the standard be equal to that of the test solution at 100% of label claims. For each drug, the chromatographic response was determined at drug concentrations corresponding to 50–150% of label claim (Table 2). The results show that linearity is adequate for the purpose of this method. For all drugs, chromatographic analysis times were less than 8 min.

Some barbiturate formulations may be stabilized with parabens (4). Relative retention times, at a column temperature of 210°C, of methyl, ethyl, and propyl paraben and benzoic acid were 0.256, 0.297, 0.388, and 0.123, respectively. The barbiturates were completely resolved from the parabens except for butabarbital and propyl paraben, where slight interference is possible. No attempt was made to determine the parabens quantitatively.

Representative dosage forms of the 7 barbiturates were assayed by the GLC and official procedures. GLC assays of tablets and capsules were done on 10 individual dosage units, as done in a content uniformity test. For other formulations, assays were done in duplicate, as were all assays by official methods. The results (Table 3) obtained by the GLC and other methods are in good agreement, and demonstrate the broad applicability of the method, both as to the number of drugs and the diversity of formulations.

REFERENCES

(1) *U.S. Pharmacopeia* (1980) 20th Rev., U.S. Pharmacopeial Convention, Rockville, MD
(2) *British Pharmaceopoeia* (1973) Her Majesty's Stationery Office, London, UK
(3) Black, D. B., Lawrence, R. C., Lovering, E. G., & Watson, J. R. (1981) *J. Pharm. Sci.* **70**, 208–211
(4) Barken, S., & Doyle, T. D. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 1022–1029
(5) Cunningham, C. G., & Barkan, S. (1975) *J. Assoc. Off. Anal. Chem.* **58**, 525–527
(6) Kaplan, G. B., & Levine, J. (1972) *J. Assoc. Off. Anal. Chem.* **55**, 152–154
(7) Budd, R. D. (1980) *J. Chromatogr.* **192**, 212–215
(8) Jaramillo, L. F., & Driscoll, J. N. (1979) *J. Chromatogr.* **186**, 637–644

(9) Vincent, F., Feuerstein, C., Gavend, M., & Faure, J. (1979) *Clin. Chim. Acta* **93**, 391–399
(10) Lau, C. E., Tang, M., & Falk, J. L. (1979) *Pharmacol. Biochem. Behav.* **11**, 355–357
(11) Hulshoff, A., Van der Houwen, O. A., Barends, D. M., & Kostenbauder, H. B. (1979) *Anal. Chim. Acta* **105**, 139–146
(12) Sun, S. R., & Hoffman, D. J. (1979) *J. Pharm. Sci.* **68**, 386–388
(13) Giovanniello, T. J., & Pecci, J. (1977) *Clin. Chem.* **23**, 2154–2155
(14) Garle, M., & Petters, I. (1977) *J. Chromatogr.* **140**, 165–169
(15) Sibert, J. L., & Fricke, F. L. (1968) *J. Assoc. Off. Anal. Chem.* **51**, 1326–1328
(16) White, P. C. (1980) *J. Chromatogr.* **200**, 271–276
(17) Tam, H. S. I., Prakongsiri, C. B., & Fine, S. L. (1981) *J. Pharm. Sci.* **70**, 783–785

Differential Pulse Polarographic Determination of Iodine in Thyroid Tablets: Collaborative Study

WALTER HOLAK
Food and Drug Administration, 850 Third Ave, Brooklyn, NY 11232

Collaborators: R. Baetz; P. Beavin; P. Gable; R. Hebert; J. Illuminati; J. Jennings, Jr; K. Panaro; T. Poplawski

A differential pulse polarographic (DPP) method for the determination of iodine in thyroid and thyroid preparations was collaboratively studied by 8 laboratories. The overall concentration of iodine in commercial thyroid tablets containing ¼, 1, 2, and 5 gr. of declared thyroid was 0.196%. The overall repeatability and reproducibility standard deviations were 0.0043 and 0.0067, respectively, and the corresponding coefficients of variation were 2.18 and 3.41%, respectively. The results obtained by the DPP method agreed with those obtained by the *U.S. Pharmacopeia* XX method at the various concentrations of declared thyroid studied. The DPP method has been adopted official first action.

The analysis of thyroid and thyroid tablets according to the *U.S. Pharmacopeia* XX (1) requires separate methods. The assay of thyroid samples involves a preliminary ashing step to release iodide, chemical transformation of iodide to iodine, and determination of iodine by titrimetry. The determination of content uniformity, on the other hand, requires oxygen flask combustion of individual tablets followed by spectrophotometry. Recently, a simple differential pulse polarographic (DPP) method for analysis of thyroid and thyroid tablets for total iodine, thyroxine, and liothyronine was reported from this laboratory (2). The present paper gives the results of a collaborative study of the procedure for total iodine, as well as those of a study performed by one of the collaborating laboratories to compare the DPP and compendial methods.

Collaborative Study

Eight collaborators from 8 different laboratories were sent detailed information and instructions on the DPP method. Each collaborating laboratory also received duplicate samples of thyroid tablets containing ¼, 1, 2, and 5 gr. of declared thyroid. The samples were randomly numbered from 1 to 8 in such a way that the presence of duplicates was not obvious to the collaborators. The collaborators were directed to analyze each sample by the proposed method, and to submit their results and worksheets to the Associate Referee.

Received August 27, 1981. Accepted October 26, 1981.
This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.
The recommendation of the Associate Referee was approved by the General Referee and Committee B and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) **65**, 486-487.

Iodine in Thyroid Tablets – Polarography

Official First Action

39.C04 ***Apparatus***

Polarograph.—Model 174 (Princeton Applied Research Corp., Princeton, NJ 08540) or equiv., with dropping Hg electrode. Typical operating parameters: scan rate 5 mV/s; scan direction "–"; potential scan range 1.5 V; initial potential −0.9 V; modulation amplitude 50 mV; differential pulse operating mode; display direction "+"; drop time, 1 s; low pass filter off; push-button, initial; offset, off; current range 1-10 μamp, or as needed.

39.C05 ***Reagents***

Use anal. reagents and glass-distd H_2O thruout.

(a) *Bromine water.*—Br-satd H_2O. Prep. fresh daily.

(b) *Potassium carbonate.*—If reagent grade K_2CO_3 gives high blank, purify as follows: Dissolve ca 200 g K_2CO_3 in 400 mL H_2O, add 50 g 20–50 mesh Amberlite IRA-400 ion exchange resin (Mallinckrodt Chemical Works), and agitate 30 min. Filter thru glass wool plug into porcelain crucible, evap. to dryness on hot plate, and heat at 675° in muffle 25 min. Cool to room temp., and grind to fine powder with mortar and pestle.

(c) *Reagent blank.*—Dissolve 8 g K_2CO_3 in ca 70 mL H_2O in 100 mL vol. flask. Add 1 mL Br-satd H_2O and 20 mg Na_2SO_3. Mix, dil. to vol. with H_2O, and mix.

(d) *Standard solns.*—(1) 1 mg I/mL: Dissolve 1.686 g KIO_3 in ca 200 mL H_2O in 1 L vol. flask. Dil. to vol. and mix. (2) 32 μgI/mL: Pipet 8 mL

0004-5756/82/6505-1059-04$01.00

std soln (*1*) into 250 mL vol. flask, dil. to vol., and mix.

(e) *Working soln.*—Pipet aliquot (*V*) of std soln (*2*) contg same amt of I contained in one tablet (see below) into 100 mL vol. flask contg 8 g K_2CO_3 dissolved in 70 mL H_2O.

Tab. strength, gr. thyroid	*I content, μg*	*Std soln 2, mL*
1/4	32.4	1
1	128.6	4
2	259.2	8
5	643.0	20

Add 1 mL Br-satd H_2O and mix. Add Na_2SO_3 (ca 20 mg) until soln becomes colorless; mix. Dil. to vol. with H_2O and mix.

39.C06 *Sample Preparation*

(a) *Composite assay.*—Weigh and finely powder ≥20 tablets. Weigh portion of powder equiv. to 1 tablet into porcelain crucible that has been washed with HNO_3 (1 + 1), rinsed with H_2O, and wiped dry. Mix with 4 g K_2CO_3 and overlay with addnl 4 g K_2CO_3. Place crucible in preheated 675° muffle 25 min. Cool, add 30 mL H_2O, carefully heat on hot plate to dissolve residue, and filter thru funnel with glass wool plug into 100 mL vol. flask. Repeat heating with 2 addnl 30 mL portions of H_2O, and add these exts to vol. flask. Add 1 ml Br-satd H_2O, mix, add Na_2SO_3 (ca 20 mg) until soln becomes colorless. Dil. to vol. with H_2O and mix.

(b) *Individual tablet assay.*—Crush 1 tablet in porcelain crucible with glass rod. Remove any sample adhering to glass rod with spatula, and add to crucible. Proceed as in Composite Assay, (a), beginning "Mix with 4 g K_2CO_3 . . ."

39.C07 *Determination*

Add ca 10 mL working soln to dry polarographic cell. Bubble N thru cell 5 min; then direct stream of N above soln. Using typical operating parameters as guide, switch selector to external cell and wait until pen becomes stationary; then depress scan button. Similarly, using same settings, analyze sample soln followed by reagent blank. From baseline established by reagent blank, measure peak hts of std and sample solns at ca −1.18 V vs SCE. Calc. as follows:

I as % of declared thyroid

$$= (PH \times V \times W_t \times 3.2)/(PH' \times W_s \times TH)$$

where PH and PH' = peak ht of sample and std, resp.; V = mL of 32 μg/mL std used to prep. working std soln; W_t and W_s = av. wt of tablet and wt of sample, g, resp.; and TH = declared thyroid per tablet, mg.

Results and Discussion

Table 1 summarizes the results submitted by the 8 collaborators on the determination of iodine in composite samples of 4 strengths of

Table 1. Collaborative results for DPP determination of iodine (%) in thyroid tablets [a]

Coll.	Declared thyroid, gr. 1/4	1	2	5
1	0.177	0.202	0.199	0.186
	0.185	0.193	0.207	0.178
2	0.194	0.201	0.190	0.196
	0.182	0.212	0.189	0.203
3	0.201	0.210	0.193	0.193
	0.198	0.203	0.195	0.193
4	0.192	0.205	0.197	0.191
	0.195	0.207	0.193	0.200
5	0.195	0.212	0.180	0.193
	0.193	0.210	0.184	0.204
6	0.189	0.206	0.196	0.196
	0.185	0.205	0.193	0.191
7 [b]	0.174	0.186	0.221	0.169
	0.200	0.200	0.221	0.173
8	0.183	0.204	0.197	0.195
	0.190	0.202	0.196	0.191

[a] Percent iodine found by USP XX method: 1/4 gr., 0.200; 1 gr., 0.218, 0.214, 0.209; 2 gr., 0.200, 0.199, 0.201; 5 gr., 0.199, 0.203, 0.202.

[b] Excluded from statistical analysis.

Table 2. Results from one collaborative laboratory for determination of iodine (%) in thyroid tablets by DPP and USP XX methods

Sample No.	Declared thyroid, gr.	Composite assay		Individual tablet assay by DPP			
		USP XX	DPP	No. of tablets assayed	Low value	High value	Av.
1	1/4	0.226	0.240	10	0.212	0.225	0.220
2	1/2	0.192	0.204	30	0.157	0.196	0.183
3	1/2	0.196	0.196	10	0.200	0.213	0.208
4	1/2	0.209	0.212	10	0.197	0.220	0.211
5	1/2	0.182	0.181	10	0.175	0.193	0.179
6	1/2	0.190	0.191	10	0.192	0.205	0.197
7	1/2	0.177	0.179	30	0.150	0.284	0.176
8	1/2	0.215	0.213	10	0.185	0.214	NS[a]
9	1	0.187	0.185	10	0.176	0.201	0.186
10	1	0.212	0.203	10	0.207	0.285	0.227
11	1	0.201	0.199	10	0.188	0.216	0.198
12	1	0.209	0.212	10	0.191	0.265	0.210
13	1	0.209	0.217	10	0.199	0.267	0.221
14	1	0.213	0.206	30	0.164	0.243	NS
15	2	0.193	0.202	10	0.196	0.206	0.199
16	2	0.212	0.211	10	0.199	0.218	0.206
17	2	0.210	0.192	10	0.197	0.216	0.208

[a] NS = not submitted.

commercial thyroid tablets by the DPP method. Statistical evaluation of these data showed that the overall mean iodine content was 0.196%. The overall repeatability and reproducibility standard deviations were 0.0043 and 0.0067, respectively, and the corresponding coefficients of variation were 2.18 and 3.41%, respectively. This evaluation does not include the results reported by Collaborator 7 because it appeared that this analyst did not add the proper amount of sodium sulfite needed to remove the excess of bromine, which is used to oxidize iodide to iodate. To avoid the future occurrence of this problem, the instructions to the proposed method were modified to read: "Add sufficient sodium sulfite (20 mg) until the solution becomes colorless."

Table 2 contains the results submitted by one of the collaborating laboratories on the analysis of composite samples of various strengths of thyroid tablets by the proposed and USP methods.

All collaborators commented favorably on the DPP method. One collaborator indicated that the USP XX method for content uniformity determination of thyroid tablets tends to be erratic and gives unreliable results. Another collaborator reported that the DPP method was more rapid and less cumbersome than its official counterpart. An additional advantage of the DPP method over the USP XX method is the reduction in the numbers of solutions and steps required for the analysis, thereby reducing the sources of error. One collaborator commented that the USP XX titrimetric method for thyroid relies on a colorless end point which is difficult to discern when tablets having a colored coating are analyzed. No such problem is encountered with the DPP method.

Table 3 presents the results for composite samples and content uniformity determinations of coated thyroid tablets with and without the coating, as reported by the collaborating laboratory that conducted the methods comparison study. In all cases, results obtained by the DPP and USP XX methods were higher when the coating was left intact than when it had been removed by rinsing.

In summary, the proposed DPP method was simpler, shorter, and more reliable; results were in close agreement with those obtained by the USP XX method. In addition, the DPP method has the added advantage that it can be applied to the composite assay as well as to individual tablets.

Recommendation

The Associate Referee recommends that the differential pulse polarographic method for thyroid and thyroid tablets be adopted official first action.

Table 3. Results from one collaborative laboratory for determination of iodine (%) in coated thyroid tablets by the DPP and USP XX methods

Sample No.	Declared thyroid, gr.	Composite assay				Individual tablet[a] assay by DPP			
		Intact coating		Coating removed		No. of tablets assayed	Low value	High value	Av.
		USP XX	DPP	USP XX	DPP				
1	1	0.247	0.254	0.231	0.233	30	0.203	0.254	NS[b]
2	1	0.240	NS	0.226	0.230	10	0.214	0.268	0.229
3	2	0.208	NS	0.193	0.209	10	0.190	0.206	0.196
4	2	0.243	NS	0.233	0.244	10	0.233	0.322	0.253
5	2	0.237	NS	0.222	0.227	10	0.191	0.233	0.214

[a] Coating removed.
[b] NS = not submitted.

Acknowledgments

The Associate Referee thanks Cesar A. Lau-Cam, Science Advisor, Food and Drug Administration, Brooklyn, NY, and Professor of Pharmacognosy, St. John's University, College of Pharmacy and Allied Health Professions, Jamaica, NY, for his invaluable assistance in the preparation of this paper, and the following collaborators, all of the Food and Drug Administration: R. Baetz, Dallas, TX; P. Beavin, Baltimore, MD; P. Gable, Cincinnati, OH; R. Hebert, New Orleans, LA; J. Illuminati, Philadelphia, PA; J. Jennings, Jr, Winchester Engineering and Analysis Center, MA; K. Panaro, Boston, MA; and T. Poplawski, New York, NY. Special thanks are due to D. Sullivan, P. Gable, and R. Carr, Food and Drug Administration, Cincinnati, OH, for supplying the data in Tables 2 and 3.

References

(1) *U.S. Pharmacopeia* (1980) 20th Rev., U.S. Pharmacopeial Convention, Rockville, MD, pp. 800–801
(2) Holak, W., & Shostak, D. (1979) *J. Pharm. Sci.* **68**, 338–342

High Performance Liquid Chromatographic Determination of Primidone in Tablets

STANLEY E. ROBERTS
Food and Drug Administration, Winchester Engineering and Analytical Center, Winchester, MA 01890

A high performance liquid chromatographic (HPLC) method is described for the quantitative determination of primidone in tablets. A ground tablet sample is diluted directly in the mobile phase, at a concentration of about 1 mg/mL of primidone, mixed and deaerated, and filtered. The resulting solution is then quantitated by HPLC. The average spike recoveries for the 50 mg and 250 mg tablets were 101.2% and 99.0%, respectively. The average recovery for an authentic mixture formulated at the 250 mg level was 100.1% with a relative standard deviation of 0.45%.

Primidone is the third most frequently administered anticonvulsant in the United States and is often given in combination with other anticonvulsants (1). A method to separate these drugs from plasma by gas chromatography with electron-capture detection has been reported (1). This method involves the extraction and derivatization of primidone with pentafluorobenzoyl chloride to form a product to which the electron-capture detector is extremely sensitive. However, the method is long and cumbersome to adapt to pharmaceutical preparations.

The ultraviolet absorption in the 250 nm region forms the basis for a number of other methods for determining primidone. The USP XX (2) procedure uses a 254–261 nm baseline for measuring the peak absorbance at 257 nm, which will somewhat reduce interference from certain ultraviolet-absorbing materials, but others will have an absorption pattern similar to that of primidone. Therefore, the method is not specific, and because the absorptivity is not high, the probability of interference from small amounts of ultraviolet-absorbing materials is increased (3).

Primidone is relatively stable due to the absence of reactive functional groups, and there are no reports of degradation under ordinary analytical conditions.

Because of its speed, simplicity, and selectivity, high performance liquid chromatography (HPLC) was selected to quantitate primidone in pharmaceutical tablet formulations. Primidone was determined in a methanol–water mobile phase at 254 nm with an octyl column. No interferences were noted.

Received December 18, 1981. Accepted March 11, 1982.

METHOD

Reagents

(a) *Solvents.*—HPLC grade water and methanol (J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

(b) *HPLC mobile phase.*—In a suitable flask, combine 500 mL water and 500 mL methanol. Filter through 0.45 μm membrane filter wetted with methanol, using magnetic stirring. Place in ultrasonic bath 10 min to deaerate.

(c) *HPLC standard solution.*—Accurately weigh 50 mg USP Reference Standard Primidone, previously dried 2 h at 105°C, and transfer to 50 mL volumetric flask. Add 35 mL mobile phase, place in ultrasonic bath 15 min, cool, and dilute to volume with mobile phase. Place in ultrasonic bath for additional 15 min and cool. Solution is stable 1 week.

Apparatus

(a) *Liquid chromatograph.*—Model ALC-202 HPLC system equipped with Model U6K injector, Model 6000A solvent delivery system, and Model 440 UV absorbance detector (Waters Associates, Inc., Milford, MA). Operating conditions: flow rate 1.0 mL/min; 254 nm detector, 0.2 AUFS; temperature, ambient; 20 μL injection.

(b) *HPLC column.*—Macherey-Nagel Nucleosil C8, 10 μm particle size, 25 cm × 3.2 mm, or equivalent.

(c) *Recorder.*—10 mV with 0.5 cm/min chart speed (Ommi-Scribe B-5000, Houston Instrument), or equivalent.

(d) *Membrane filters.*—Nylon-66, pore size 0.45 μm (Rainin Instrument Co., Woburn, MA 01890), or equivalent.

Preparation of Samples

Determine average weight of 20 tablets and grind to pass No. 60 sieve. Transfer accurately

weighed portion of the powder equivalent to 50 mg primidone to 50 mL volumetric flask. Add 35 mL mobile phase, place in ultrasonic bath 15 min, cool, and dilute to volume with mobile phase. Place in ultrasonic bath additional 15 min and cool. Filter solution through 0.45 μm membrane filter and use this solution as sample preparation.

Determination

Equilibrate system with column in instrument and mobile phase set at flow rate of 1.0 mL/min. Inject 20 μL HPLC standard solution and adjust flow rate and sensitivity so that peak response is ca 45% full scale with retention time of ca 3 min. In suitable system, coefficient of variation (CV) of the peak responses of 5 replicate injections is ≤2.0%. Proceed with sample analysis, using 20 μL injections for each standard and sample solution.

Calculations

Determine peak responses of standard and sample peaks and calculate amount of primidone in tablets:

mg Primidone/tablet

$$= (R/R') \times 50\,(C)\,(T)/W$$

where R and R' are peak response of sample and standard solutions, respectively; C is concentration of primidone standard solution in mg/mL; T is average tablet weight in mg; and W is sample weight in mg.

Results and Discussion

Under the HPLC conditions described, primidone has an elution time of about 3 min at a flow rate of 1.0 mL/min. Ten replicate injections of the standard solution gave a CV of 0.651%. Seven injections, in triplicate, of varying volumes of standard solution representing approximately 5, 10, 15, 20, 25, 30, and 40 μg primidone were introduced into the liquid chromatograph, the average response was plotted for each volume, and the linearity was calculated. The correlation coefficient was 0.9994 with an intercept of zero and the CV averaged 1.40% over the entire concentration range. The method therefore was determined to be reproducible and linear throughout this range. A typical chromatogram is shown in Figure 1.

Spike recoveries through the proposed method were determined at the 50 mg and 250 mg/tablet level and averaged 100.1% with a relative standard deviation (SD) of 1.25% (Table 1). Also, an authentic mixture was analyzed at the 250 mg level with an average recovery of 100.1% and a relative SD of 0.45% (Table 2). Excipients were tested separately from commercial tablet formulations. No interferences were detected. Primidone is completely soluble in the mobile phase at the concentrations used. It shows no degradation in the mobile phase for at least 1

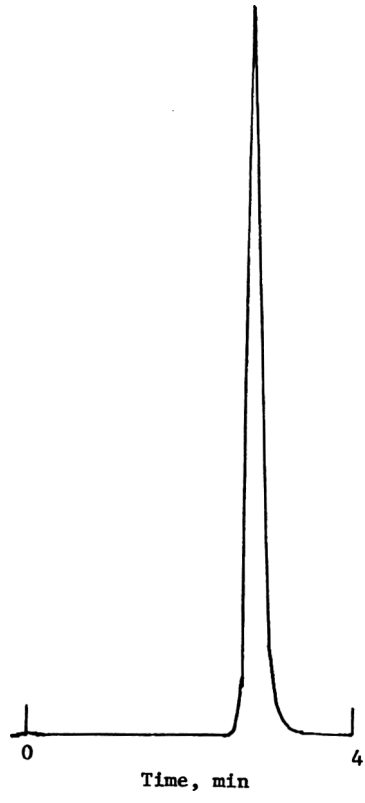


Figure 1. A typical chromatogram of a primidone tablet sample, 1 mg/mL.

Table 1. Recoveries of fortified primidone samples through HPLC method

Sample	Primidone added, mg	Primidone recovered, mg	Primidone recovered, %
Reagent blank	0	0	—
Tablets, 50 mg	25.4	25.7	101.2
	26.1	26.4	101.1
Tablets, 250 mg	25.3	25.1	99.2
	25.3	25.0	98.8
Av.			100.1
SD			1.25
CV, %			1.25

Table 2. Recoveries of authentic mixture of primidone through HPLC method

Sample	Primidone added, mg	Primidone recovered	
		mg	%
Reagent blank	0	0	—
Authentic mixture	250	250.3	100.1
	250	251.3	100.5
	250	249.0	99.6
Av.			100.1
SD			0.45
CV, %			0.45

Table 3. Comparison of precision of USP XX and HPLC methods for commercial primidone tablets

Primidone declared, mg/tab.	Primidone found			
	USP XX method		HPLC method	
	mg/tab.	%	mg/tab.	%
50	51.5	103.0	50.0	100.0
	51.4	102.8	50.7	101.4
250	248.8	99.5	256.6	102.6
	254.9	102.0	254.5	101.8
Mean		101.8		101.5
SD		1.61		1.09
CV, %		1.58		1.07

week in clear glass containers at room temperature and under normal laboratory lighting.

A comparison of results of actual tablet analyses by the USP XX and the HPLC methods is shown in Table 3. The analyses were performed on each of 2 tablet samples at 2 concentration levels. The results of the 2 methods are comparable but the precision of the HPLC method appears to be better.

Acknowledgment

The assistance of Michael F. Delaney, WEAC Science Advisor, in editing this manuscript is acknowledged.

References

(1) Wallace, J. E., Hamilton, H. E., Shimek, E. L., Schwertner, H. A., & Blum, K. (1977) *Anal. Chem.* **49**, 903

(2) *U.S. Pharmacopeia* (1980) 20th Rev., U.S. Pharmacopeial Convention, Rockville, MD, pp. 657–658

(3) Daley, R. D., & Florey, K. (Eds) (1973) *Analytical Profiles of Drug Substances*, Vol. 2, Academic Press, New York, NY, pp. 409–433

ALCOHOLIC BEVERAGES

Gas Chromatographic Determination of Primary and Secondary Amines as Pentafluorobenzamide Derivatives

BRIAN D. RIPLEY, BRUCE J. FRENCH,[1] and LLOYD V. EDGINGTON[2]
Ontario Ministry of Agriculture and Food, Provincial Pesticide Residue Testing Laboratory, c/o University of Guelph, Guelph, Ontario, Canada N1G 2W1

An analytical method is described for the determination of mono- and dialkylamines in foodstuffs. Amines are derivatized to their pentafluorobenzamides which may be separated by gas chromatography (GC) and determined using an N-P detector. Analysis of the derivatives by GC-mass spectrometry indicated they were all mono-substituted. The amines were isolated from foodstuffs by alkaline distillation of ≥75% sample volume. The distribution of dimethylamine (DMA) in distillate volumes indicated 2 maxima from barley and malt, which could represent 2 or more sources of DMA. DMA concentrations of 6.6–8.8 ppm in barley, 11 ppm in malt, and 1.2 ppm in beer are higher than previously reported.

N-Nitroso compounds have been shown to be carcinogenic (1, 2) and many *N*-nitrosamines have been found in or are formed during the processing of foodstuffs, fish, and malt beverages (3–8); *N*-nitrosamines can also be formed in vivo (6–9). Several investigators have focused attention on the presence of nitrosamine precursors such as nitrates, nitrites, and secondary amines in food commodities (10–15). Many amines such as methylamine (MA), dimethylamine (DMA), and trimethylamine (TMA) are ubiquitous in the environment (8, 16).

Direct gas chromatography of alkylamines is often difficult because many column packing materials tend to absorb the polar amines and cause peak tailing and reduced sensitivities. Some success has been achieved with a variety of different gas chromatographic supports and liquid phases. For example, alkali-treated supports (17–19), alkali precolumns (20), porous polymer packings such as Chromosorb 103 (21) or Porapaks (22), and Graphon coated with a light load of liquid phase such as tetraethylenepentamine (TEP) (11, 23–25) have been examined for amine analysis. Despite a low column temperature, resolution of many of the low boiling point alkylamines is less than ideal; furthermore, many studies relate only to MA, DMA, and TMA (15, 25). The volatile amines must be handled carefully, and are usually determined as their salts or with the aid of cryogenic traps to avoid losses during quantitative analyses.

Chemical derivatization offers an alternative means of handling chemically and thermally unstable compounds as well as many other types of compounds not amenable to direct gas chromatography (GC). The resultant derivatives usually exhibit good peak shape and sensitivity while the higher molecular weight is useful for mass spectroscopic analysis. A variety of different derivatization procedures have been used to determine amines by GC or thin layer chromatography. Typical processes involve benzylation (26), dansylation (12, 21, 27), dinitrophenylation (28–31), tosylation (13, 14), and trifluoroacetylation (16). Bradway and Shafik (32) discuss the formation of these derivatives for GC determination of anilines.

An alternative derivatization procedure for amines is benzoylation. Lodge and Barber (33) prepared benzamides to determine aliphatic amines in air by paper chromatography. Tilden and Van Middelem (34) examined aniline, amide, sulfonamide, and benzamide derivatives of methyl- and dimethylamine and found the 4-bromo-*N*-methylbenzamide derivatives to be superior from the point of view of ease of preparation, thermal and chemical stability during GC analysis, and sensitivity to electron capture detection. Benzoylation is often applied to the derivatization of pesticide residues with reactive NH moieties.

This paper discusses the derivatization of mono- and dialkylamines by using pentafluorobenzoyl chloride in a Schotten–Baumann type reaction and the gas chromatographic and mass spectroscopic analysis of the pentafluorobenzamides. The application of this procedure to the determination of primary and secondary amines, and in particular dimethylamine, in barley and other substrates is also examined.

[1] Present address: Canada Malting Co., Ltd, 369 Lakeshore Blvd E, Toronto, Ontario, Canada N5A 1C2.
[2] University of Guelph, Department of Environmental Biology, Guelph, Ontario, Canada N1G 2W1.

0004-5756/82/6505-1066-07$01.00

Experimental

Apparatus and Reagents

(a) *Gas chromatography.*—Hewlett-Packard Model 5710 equipped with N-P detector and 1.84 m × 2 mm id glass column packed with Ultra-Bond, 100–120 mesh (RFR Corp., Hope, RI 02831). Operating conditions: temperatures (°C)—column 120, detector 300; gas flows (mL/min)—helium carrier 30, hydrogen 3.0, air 50. Voltage to detector was adjusted for ca 10% offset at attenuation 32. Under these conditions, 100 ng DMA-derivative produced 50% FSD on Hewlett-Packard Model 3380A reporting integrator at attenuation 256 and exhibited a retention time of ca 1.7 min.

(b) *Mass spectrometer.*—Hewlett-Packard Model 5992 GC-MS equipped with 0.92 m × 2 mm id glass column packed with Ultra-Bond or 2% OV-101, jet separator, and Model 9885 disk system.

(c) *Amine standards.*—All amine standards were reagent grade (Eastman Organic Chemicals or Fisher Scientific Ltd). Gramine (3-[dimethylaminomethyl]indole) was obtained from Sigma Chemical Co., St. Louis, MO 63118, and *N,N*-dimethyltryptamine was supplied by the Bureau of Dangerous Drugs, Health and Welfare, Ottawa, Canada. The lower alkylamines were obtained as their hydrochloride salts, and all stock and working solutions were prepared in 0.1N HCl.

(d) *Pentafluorobenzoyl chloride solution.*—Dissolve 2 mL reagent (PCR Research Chemicals, Inc., Gainesville, FL 32602, or Aldrich Chemical Co., Inc., Milwaukee, WI 53233) in 100 mL benzene.

Distillation

Place sample (typically 10 g grain, 1 g fungal teliospores) in 250–500 mL round-bottom flask and add 70 mL 50% NaOH plus 100 mL water; add only 70 mL NaOH to 100 mL beer. Connect round-bottom flask on heating mantle to Liebig condenser with 70° connecting tube and receiving adapter. Collect 125 mL distillate in 250 mL graduated Erlenmeyer flask containing 5 mL 1N HCl. During first part of distillation, a lower heat results in effective loss of substrate structural integrity and reduced foaming; typically 1–2 h is required to collect 125 mL distillate.

Derivatization

To acidic distillate, add 9.0 mL benzene, 5 mL 2M K_2CO_3, and 1.0 mL pentafluorobenzoyl chloride solution. Stopper and shake 10 min on wrist-action shaker. Transfer to 250 mL separatory funnel and remove supernate for GC analysis.

Results and Discussion

Mono- and dialkylamines readily react with pentafluorobenzoyl chloride in aqueous solution containing a trace of K_2CO_3. Yield of the pentafluorobenzamides was maximum within 10 min as observed previously (34). A macro scale synthesis indicated immediate formation of the derivative with the drop-wise addition of pentafluorobenzoyl chloride. Pentafluoro-*N,N*-dimethylbenzamide (DMA-PFB) could not be recrystallized from alcohol; the derivative was extracted from water with benzene, and the benzene was evaporated with nitrogen. The oily residue was used for standard solutions and recovery studies.

Excess reagent was required for optimum derivatization of DMA in 125 mL distillate. Furthermore, because of the presence of many endogenous amines in the substrate, more was required for quantitative derivatization. It was determined that with 10 g barley, the equivalent of 20 mg pentafluorobenzoyl chloride produced consistent, maximum recoveries. The amount of reagent required will, therefore, vary with the amount and type of substrate.

The pentafluorobenzamides may be qualitated and quantitated by gas chromatography (GC). Initial attempts to determine low levels of DMA-PFB by using ^{63}Ni electron capture detection were unsuccessful because of a large reagent blank peak at the retention time of DMA-PFB on 2% OV-101. Tilden and Van Middelem (34) observed a similar effect with other halogenated benzoyl chloride reagents. Attempts at cleanup and fractionation of the interference on silicic acid (35) or silica gel (36) failed. With an N-P detector, the reagent blank showed no extraneous peaks and the pentafluorobenzamides had linear response characteristics and good sensitivity.

One consideration in using the N-P detector was the effect of excess reagent on the detector alkali bead. Chlorinated solvents may cause reversible sensitivity loss and contamination of the alkali source with chloride ions; silanizing reagents may also coat the source (37). Once the GC system was equilibrated by repeated injections of derivatized standards, it performed well for over 6 months without changes in columns or collectors; voltage to the collector was left on overnight and the offset was adjusted to about 10% (×32) as required. Glass wool in the injec-

Table 1. Retention times (RRT) of pentafluorobenzoyl (PFB) derivatives relative to *N*-methylamine-PFB on 3 GC columns

Compound	RRT		
	Ultra-Bond[a]	2% OV-101 on Ultra-Bond[b]	2% OV-101 on Chrom. W, HP[c]
N,N-Dimethylamine	0.24	0.35	0.88
N-Methyl-*N*-ethylamine	0.28	0.49	1.21
N,N-Diethylamine	0.33	0.51	1.59
N,N-Diisopropylamine	0.40	0.89	2.40
N,N-Di-*n*-propylamine	0.62	1.17	3.53
N-Isopropylamine	0.71	0.88	1.33
Pyrrolidine	0.94	1.63	3.73
N-Ethylamine	0.94	0.92	1.24
N,N-Diisobutylamine	0.95	1.91	5.71
N-Methylamine	1.00[a]	1.00[b]	1.00[c]
Piperidine	1.02	2.02	4.89
N-Propylamine	1.40	1.51	2.04
N,N-Di-*n*-butylamine	1.41	2.71	8.51
N-Isobutylamine	1.65	1.72	2.66
N-Butylamine	2.27	2.65	3.42
N-Pentylamine	3.88	3.98	5.84

[a] 1.84 m × 2 mm id, 120°C, MA-PFB = 7.2 min.
[b] 0.61 m × 2 mm id, 120°C, MA-PFB = 3.4 min.
[c] 1.23 m × 2 mm id, 120°C, MA-PFB = 2.9 min.

tion area required periodic replacement as indicated by peak tailing and reduced sensitivity. A collaborative detector study was conducted on the system after several months of continued use, with excellent results; only the N-P selectivity changed slightly to greater P response. Samples examined with a flame photometric detector (P-mode) showed no peaks.

In the qualitative analysis of these amines, it is important that the GC peaks be resolved, particularly for those amines from endogenous sources (25). Initial attempts with a column of 2% OV-101 on Chromosorb W indicated that the derivatized monoalkylamine homologs were separated; however, MA-PFB and DMA-PFB had similar retention times and the preponderance of MA in samples overshadowed the DMA-PFB. The Ultra-Bond column successfully resolved DMA-PFB from the later-eluting MA-PFB (Table 1; Figure 1).

Gas chromatography–mass spectrometry (GC–MS) of the higher molecular weight derivatives is easily accomplished using a low resolution mass spectrometer. All derivatives were examined by GC–MS and were mono-substituted. The base peak in all cases was m/z 195, representing the pentafluorobenzoyl (PFB) group; common fragment ions from PFB were observed at m/z 167 (—CO), 148, 136, and 117 (Figure 2). The derivatives exhibited the expected molecular ion although all ion intensities above m/z 195 were weak (<25%). The derivatives fragmented as expected for the alkyl substituents (Table 2), and the lower alkyl derivatives also showed loss of fluorine (M − 19). Full scan or selected-ion-monitoring of m/z 195 and the molecular ion of the derivatized amine constitutes confirmation.

A typical method of isolating the volatile amines from food substrates involves base hydrolysis, distillation, and trapping of the evolved amines as their hydrochloride salts (13, 15, 16, 21); our previous experience (38) indicated this was a viable procedure. Volume of distillate required for quantitative recovery of the amines remained in question because rapid evolution of amines from pesticide sources (35, 39), distillation to both one-half volume (15) and equal volume (21) of sample solution had been reported. Typical distillation recovery curves are shown in Figures 3 and 4. More than 90% DMA standard was distilled in the first 10 mL; recovery plateaued at 20 mL with 95% recovery. Under the described hydrolysis conditions, most of the endogenous DMA was distilled after 125 mL of a 170 mL sample volume. Further distillation was impractical because of charring. Doubling the initial sample volume resulted in a similar distillation curve but with the abscissa values approximately doubled. With increasing sample volumes (7:10 $NaOH:H_2O$), essentially the same total DMA was found after 75% sample distillation, while MA levels, presumably from proteins, were still increasing and other less volatile

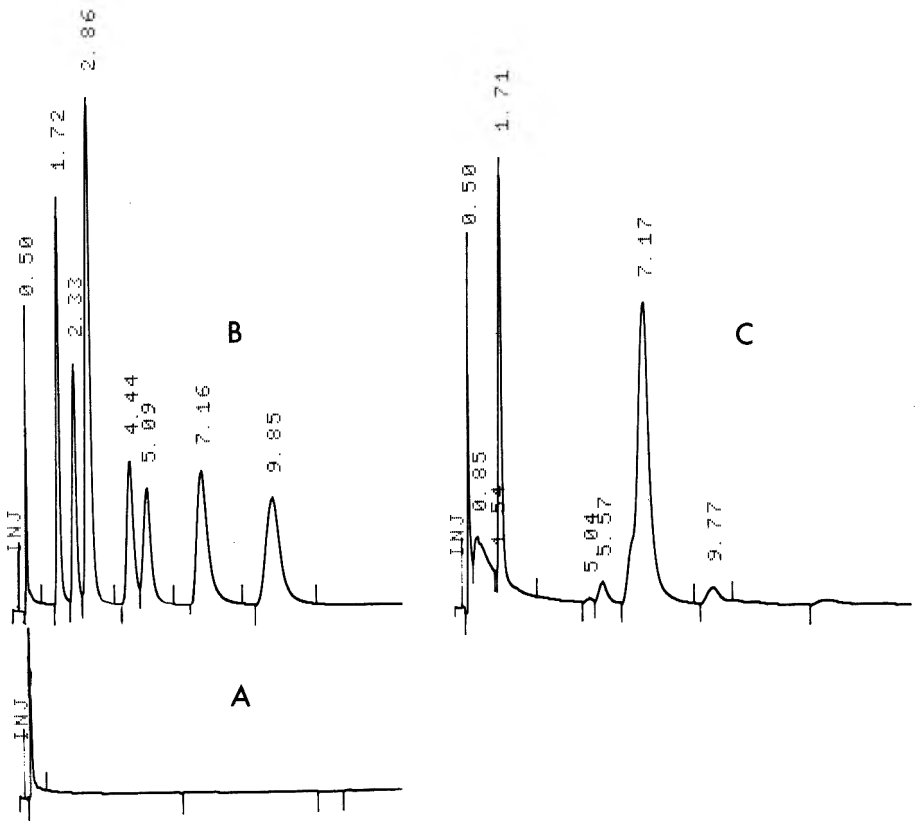


Figure 1. Gas chromatograms of (A) reagent blank, (B) amine standard solution, and (C) 1 g barley/mL. Operating conditions given in text; 5 μL injection, Ultra-Bond column, 256 attenuation. Peak identity (ng): dimethylamine, 1.72 min (25), diethylamine (25), diisopropylamine (50), di-*n*-propylamine (50), isopropylamine (50), methylamine, 7.16 min (100), and propylamine (100).

amines tended to appear in later volumes. A sample was distilled as described and then 125 mL water was re-introduced by dropping funnel and a further 10 mL distillate was collected. Very little DMA was recovered in the second distillate, whereas MA levels continued to increase.

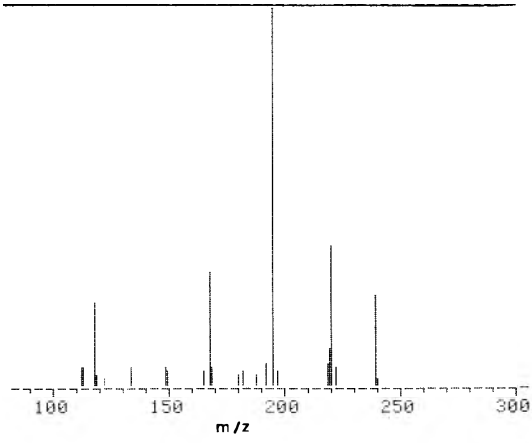


Figure 2. GC-mass spectrum of pentafluorobenzoyl derivative of dimethylamine.

Because all substrates tested contained DMA and evolution of DMA standard was rapid, no other recovery studies were conducted. From Figure 3, it appears that most of the DMA in a barley sample is recovered in the first 125 mL distillate. A similar distillation curve was obtained with teliospores of *Ustilago nuda* (loose smut of barley) as the substrate. Malted samples indicated a much earlier release of some DMA (Figure 4). The 2 apparent maxima in the distillation curves might indicate several sources of DMA, i.e., a somewhat volatile or freer DMA and a "bound" endogenous or conjugated source. In all cases, complete recovery of DMA appeared to be obtained after distillation of 75% of the sample volume; incomplete distillation of DMA in other studies may account for the lower concentrations of DMA reported.

Table 2. Mass spectral data for pentafluorobenzamide derivatives. Common fragment ions from pentafluorobenzoyl moiety occur at m/z 195 (base peak), 167, 148, 136, and 117

Derivative	m/z	
	Molecular ion	Fragment ions
N,N-Dimethylamine	239	220
N,N-Diethylamine	267	252, 248, 232, 224
N,N-Di-*n*-propylamine	295	281, 276, 266, 246, 224
N,N-Diisopropylamine	295	280, 260, 252, 238
N,N-Di-*n*-butylamine	323	280, 259, 238, 220
N,N-Diisobutylamine	323	280, 224
N-Methyl-*N*-ethylamine	253	238, 234, 218
N-Methylamine	225	206
N-Ethylamine	239	224, 220
N-Propylamine	253	238, 207
N-Isopropylamine	253	238, 234, 218, 212
N-Butylamine	267	252, 248, 238, 224, 212
N-Isobutylamine	267	252, 224, 219, 212
Pyrrolidine	265	221
Piperidine	279	260

Whether DMA occurs freely or as part of a natural product or other complex is uncertain. Several dimethylamine-containing pesticides and natural products were examined for recovery with the described procedure. Recovery of DMA after hydrolysis–derivatization was quantitative with the pesticides and *N,N*-dimethyltyrptamine. Gramine showed complete conversion to DMA-PFB with only the derivatization step; hordenine (*p*-[2-dimethylaminoethyl]phenol; *N,N*-dimethyltyramine) was unavailable for testing. Gramine (40) and hordenine (41, 42) are known to be natural products formed during the germination of barley and may be found in malted beverages (43); furthermore, these alkaloids have been implicated as the precursors of *N*-nitrosodimethylamine (NDMA) in direct-fired malt (44, 45). Coffin (46) showed that hordenine was not derivatized with trifluoroacetic anhydride, and Gyllenhaal et al. (47) used hordenine sulfate to remove excess pentafluorobenzyl bromide via the *O*-derivative in a fatty acid derivatization method. The somewhat quicker hydrolysis–distillation of DMA from malt samples (Figure 4) might indicate this other source. Several samples were shaken with 0.1N HCl and the filtrate was derivatized while the residue was hydrolyzed and derivatized. The total DMA from these fractions compared favorably with the complete hydrolysis procedure, although the former was lower in all cases. With barley, no DMA was found in the acid, whereas malted samples indicated up to 25% of the total DMA was acid-extractable. Various parts of the malt were similarly analyzed and most of the acid-extractable DMA appeared to originate in the roots (48). O'Brien et al. (49) recently reported that the highest concentration of NDMA in unsulfured malt fractions occurred in the rootlets and husks. Malt samples contained more DMA than did the barley from which it was produced.

Four replicate analyses on 2 different barley samples indicated DMA concentrations of 7.1 ± 0.5 and 7.6 ± 0.3 ppm. DMA was present in all barley samples at concentrations ranging from 6.6 to 8.8 ppm. These levels are greater than the 0.5 ppm DMA reported by Neurath and Schreiber (16). Analysis of a Canadian beer sample indicated 1.2 ppm DMA while malt samples contained a mean concentration of 11 ppm DMA

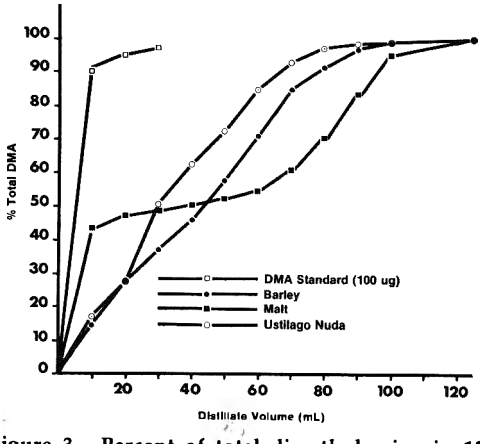


Figure 3. Percent of total dimethylamine in 125 mL distillate found after various volumes.

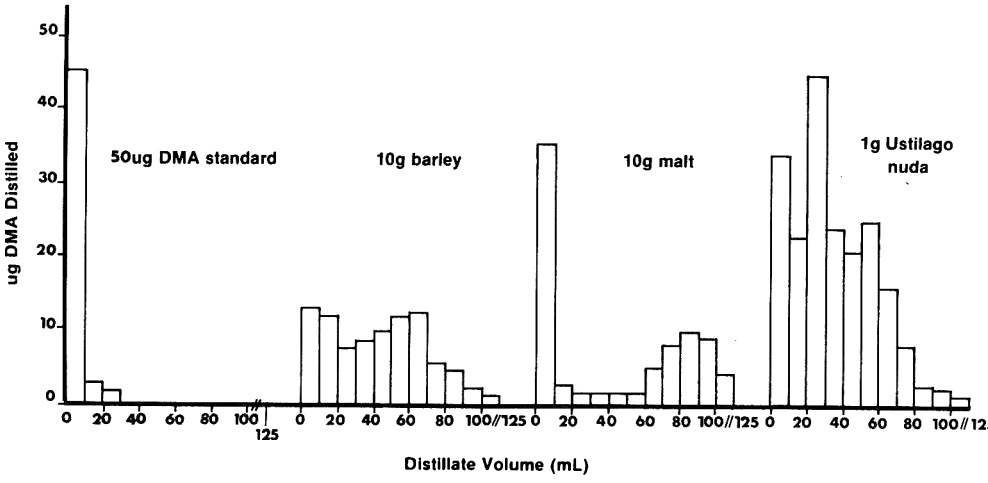


Figure 4. Distribution of dimethylamine in various sample distillation volumes.

on a dry basis; the malt concentrations are again higher than those reported by Neurath and Schreiber (16), but the levels in beer are only slightly higher than the 0.07–0.7 ppm range reported by Singer and Lijinsky (13), Spiegelhalder et al. (45), and Slaughter and Uvgard (50) for European and American beers. In fermented beverages from Nigeria, concentrations of 4.6–31.6 ppm DMA have been found (15). Zee et al. (51) recently determined other biogenic amines in beer at concentrations higher than previously reported.

DMA and MA appeared to be ubiquitous in all samples, and other amines were also present in various amounts. Variations of several parts per million DMA were found between cultivars of barley. Some substrates, such as cereals, may be classified by the amines present. This method appears to be satisfactory for the quantitative determination of DMA and also for the qualitative analysis and mass spectrometric confirmation of other endogenous amines in different substrates.

Acknowledgment

The authors thank the Brewer's Association of Canada and the Ontario Ministry of Agriculture and Food for support.

References

(1) Magee, P. N., & Barnes, J. M. (1956) *Brit. J. Cancer* **10,** 114–122
(2) Magee, P. N., & Barnes, J. M. (1967) *Adv. Cancer Res.* **10,** 163–246
(3) Bogovski, P., Preussmann, R., & Walker, E. A., (Eds.) (1972) *N-Nitroso Compounds, Analysis and Formation*, International Agency for Research on Cancer, Scientific Publication 3, Lyon, France
(4) Bogovski, P., & Walker, E. A. (Eds.) (1975) *N-Nitroso Compounds in the Environment*, International Agency for Research on Cancer, Scientific Publication 9, Lyon, France
(5) Scanlan, R. A. (1975) *Crit. Rev. Food Technol.* **5,** 357–402
(6) Crosby, N. T. (1976) *Residue Rev.* **64,** 77–135
(7) Fishbein, L. (1979) *Sci. Total Environ.* **13,** 157–188
(8) Fine, D. H. (1980) *Adv. Environ. Sci. Technol.* **10,** 39–123
(9) Sander, J., Schweinsberg, F., & Menz, H. P. (1968) *Z. Physiol. Chem.* **349,** 1691–1697
(10) Fazio, T., Damico, J. N., Howard, J. W., White, R. H., & Watts, J. O. (1971) *J. Agric. Food Chem.* **19,** 250–253
(11) Miller, A., III, Scanlan, R. A., Lee, J. S., & Libbey, L. M. (1972) *J. Agric. Food Chem.* **20,** 709–711
(12) Spinelli, A. M., Lakritz, L., & Wasserman, A. E. (1974) *J. Agric. Food Chem.* **22,** 1026–1029
(13) Singer, G. M., & Lijinsky, W. (1976) *J. Agric. Food Chem.* **24,** 550–553
(14) Singer, G. M., & Lijinsky, W. (1976) *J. Agric. Food Chem.* **24,** 553–555
(15) Bassir, O., & Maduagwu, E. N. (1978) *J. Agric. Food Chem.* **26,** 200–203
(16) Neurath, G. B., & Schreiber, O. (1975) in *Nitroso Compounds in the Environment*, P. Bogovski & E. A. Walker (Eds.), International Agency for Research on Cancer, Scientific Publication 9, Lyon, France, pp. 211–214
(17) James, A. T. (1952) *Biochem. J.* **52,** 242–247
(18) James, A. T., Martin, A. J. P., & Smith, G. H. (1952) *Biochem J.* **52,** 238–242
(19) Smith, E. D., & Radford, R. D. (1961) *Anal. Chem.* **33,** 1160–1162
(20) Umbreit, G. R., Nygren, R. E., & Testa, A. J. (1969) *J. Chromatogr.* **43,** 25–32
(21) Gruger, E. H., Jr (1972) *J. Agric. Food Chem.* **20,** 781–785

(22) Lindsay Smith, J. R., & Waddington, D. J. (1968) *Anal. Chem.* **40,** 522–527
(23) Sze, Y. L., Borke, M. L., & Ottenstein, D. M. (1963) *Anal. Chem.* **35,** 240–242
(24) Di Corcia, A., Fritz, D., & Bruner, F. (1970) *Anal. Chem.* **42,** 1500–1504
(25) Dunn, S. R., Simenhoff, M. L., & Wesson, L. G., Jr (1976) *Anal. Chem.* **48,** 41–44
(26) Moffat, A. C., Horning, E. C., Matin, S. B., & Rowland, M. (1972) *J. Chromatogr.* **66,** 255–260
(27) Frei, R. W., & Lawrence, J. F. (1972) *J. Assoc. Off. Anal. Chem.* **55,** 1259–1264
(28) Day, E. W., Jr, Golab, T., & Koons, J. R. (1966) *Anal. Chem.* **38,** 1053–1057
(29) Crosby, D. G., & Bowers, J. B. (1968) *J. Agric. Food Chem.* **16,** 839–843
(30) Holden, E. R., Jones, W. M., & Beroza, M. (1969) *J. Agric. Food Chem.* **17,** 56–59
(31) Cohen, I. H., & Wheals, B. B. (1969) *J. Chromatogr.* **43,** 233–240
(32) Bradway, D. E., & Shafik, T. (1977) *J. Chromatogr. Sci.* **15,** 322–328
(33) Lodge, J. P., Jr, & Barber, E. D. (1961) *Anal. Chim. Acta* **24,** 235–240
(34) Tilden, R. L., & Van Middelem, C. H. (1970) *J. Agric. Food Chem.* **18,** 154–158
(35) Newsome, W. H. (1980) *J. Agric. Food Chem.* **28,** 319–321
(36) Coburn, J. A., Ripley, B. D., & Chau, A. S. Y. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 188–196
(37) Hewlett-Packard (1978) Operating note 5950-3535
(38) Ripley, B. D., Lougheed, E. C., & Miller, S. R. (1979) *J. Agric. Food Chem.* **27,** 1389–1391
(39) Ferguson, C. E., & Gard, L. (1969) *J. Agric. Food Chem.* **17,** 1062–1065
(40) Mudd, S. H. (1961) *Nature (London)* **189,** 489
(41) Mann, J. D., Steinhart, C. E., & Mudd, S. H. (1963) *J. Biol. Chem.* **238,** 676–681
(42) Leete, E., Kirkwood, S., & Marion, L. (1952) *Can. J. Chem.* **30,** 749–760
(43) Kahn, J. H. (1969) *J. Assoc. Off. Anal. Chem.* **52,** 1166–1178
(44) Widmaier, R. (1978) paper presented at International Conference on Nitrosamines, Montreal, Quebec, Canada, Sept. 16–18
(45) Spiegelhalder, B., Eisenbrand, G., & Preussmann, R. (1979) *Food Cosmet. Toxicol.* **17,** 29–31
(46) Coffin, D. E. (1969) *J. Assoc. Off. Anal. Chem.* **52,** 1044–1047
(47) Gyllenhaal, O., Brötell, H., & Hartvig, P. (1976) *J. Chromatogr.* **129,** 295–302
(48) French, B. J., Ripley, B. D., & Edgington, L. V. (1982) accepted by *Master Brew. Assoc. Am. Tech. Q.*
(49) O'Brien, T. J., Lukes, B. K., & Scanlan, R. A. (1980) *Master Brew. Assoc. Am. Tech. Q.* **17,** 196–200
(50) Slaughter, J. C., & Uvgard, A. R. A. (1971) *J. Int. Brew. London* **77,** 446–450
(51) Zee, J. A., Simard, R. E., & Desmarais, M. (1981) *Can. Inst. Food Sci. Technol. J.* **14,** 119–122

FEEDS

Crude Fiber Determination Using Ceramic Fiber to Replace Asbestos

RANDY L. KNOX, DAVID S. ENGVALL, and BURTON E. GINTHER
Washington Department of Agriculture, 2017 S 1st St, Yakima, WA 98903

Crude fiber was determined in a wide range of feed products by a method which specifies ceramic fiber as a filter medium instead of the more hazardous and difficult to obtain asbestos. Results correlated well with those obtained by using AOAC official final action method 7.061-7.065 (correlation coefficient, 0.9994). For 8 samples, the coefficients of variation ranged from 0.74 to 4.80%. Compared with the AOAC method, the proposed method showed a slight negative bias of 0.1%. Compared with asbestos, ceramic fiber was easier to prepare for use, filtering was faster, and samples bumped less.

Asbestos fiber has had long use as a filter medium and as a filter aid in crude fiber determination. Its use is specified in the AOAC official final action method, **7.061-7.065** (1). Because asbestos is a known carcinogen (2), an adequate replacement is desirable. The AOAC asbestos-free method, **7.066-7.068**, has limited use, primarily because the filtration step is slow. We attempted the use of glass fiber (GFA) paper in a manner similar to that of Craddick and Hatfield (3); the paper clogged, resulting in a slow filtration.

We found that a ceramic fiber which is often used for high temperature insulation can replace asbestos in crude fiber determinations with minor variations in the official method, **7.061-7.065.** There are several advantages to using ceramic fiber: It is not known to have the health hazards of asbestos fiber. It does not form an air suspension as easily as does asbestos, which should limit any possible harmful effects caused by inhalation and deposition in the lungs. Furthermore, the modified method allows rapid filtration and washing, and minimizes bumping of the digestion mixture.

This paper reports the comparison of the modified (ceramic fiber) method with the AOAC method which uses asbestos, **7.061-7.065.**

METHOD

Reagents

(a) *Sulfuric acid solution.*—0.255 ± 0.005N. Check concentration by titration.

(b) *Sodium hydroxide solution.*—0.313 ± 0.005N, free or nearly free of sodium carbonate. Check concentration by titration.

(c) *Ceramic fiber.*—Cerafiber, 8 lb/cu. ft (E. J. Bartell, 700 Powell Ave, SW, Renton, WA 98055). Blend ca 10 g ceramic fiber with 1 L water for minimum time necessary to achieve a pourable suspension, ca 5-10 s. This fiber can be re-used as long as it will form a cohesive mat. Filter to remove water.

(d) *Alcohol.*—Anhydrous, 3A (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(e) *Antifoam.*—Dilute SWS 213 (Stauffer Chemical Co., Adrian, MI 49221) with water (1 + 2) and suspend by shaking.

Apparatus

Use **7.063(a)**, **(b)**, (c), and **(f)**, and modify **(d)** and (e) (ref. 1) as follows:

(d) *Filtering device.*—Polypropylene Buchner funnel, 42.5 mm, Nalgene 7280-0425 (VWR Scientific, Inc., PO Box 3551, Seattle, WA 98124) modified with 400 mesh stainless steel screen sealed over filtering surface as described in **7.063**(d). Further modify device by extending funnel sides with standard $1\frac{1}{2}$ in. SCH 40 PVC pipe coupling so that screen-to-rim distance is ca 7 cm.

(e) *Suction filter.*—To accommodate filtering device, attach suction flask to vacuum source through valve which functions to break vacuum. Put two T connections in line, one connected to vacuum gauge measuring **0**-30 in. Hg and the second to a valve to the atmosphere, which is used to control vacuum.

Determination

Extract 2 g sample or blank containing no sample in porcelain thimble with ether as described in **7.055-7.056** (1). Add ca 1 g prepared ceramic fiber to 600 mL beaker containing 200 mL 0.255N H_2SO_4. Bring solution to boil on digestion apparatus. Remove beaker containing boiling solution, and add ether-free extracted sample and one drop of antifoam. Add more antifoam later if necessary to prevent excessive foaming. Bring sample to boil and boil gently

Received February 2, 1982. Accepted March 5, 1982.

0004-5756/82/6505-1073-03$01.00

exactly 30 min. Additional samples may be added to other beakers in sequence at 2.25 min intervals. Occasionally, swirl beakers to minimize foaming and prevent adhesion of sample to beaker sides.

Prepare filter pad by resuspending prepared ceramic fiber in water. Then pour into filtering device with vacuum disconnected. Slowly turn on vacuum valve while distributing fiber to form uniform pad. Adjust vacuum to 2–4 in. Hg by using second valve. At end of 30 min boiling, filter hot acid mixture through ceramic fiber pad. Adjust vacuum to that needed to maintain filtration rate which lets rinses completely cover and rinse sample. Use 50–75 mL near-boiling water to rinse any remaining sample from beaker into filtering device. Repeat with three 50 mL portions of hot water. Increase vacuum to suck pad dry.

Use forceps to transfer pad with sample back to beaker. Add 200 mL near-boiling 0.313N NaOH. Bring to boil on digestion apparatus and boil gently 30 min. During this period, remove additional samples from apparatus, filter through prepared pads, and begin basic digestion at 2.25 min intervals.

Prepare ceramic fiber filter pad, and, at end of 30 min basic digestion, filter hot digest through the pad as previously described. Rinse contents from beaker with minimum volume of hot water. Use valve to nearly turn off vacuum to filtering device while adding to it 25 mL near-boiling 0.255N H_2SO_4. Then increase vacuum to allow filtration rate which lets rinses completely cover and rinse sample. Rinse filter pad 3 times with ca 50 mL hot water. Increase vacuum to remove extra water. Turn off vacuum, add 25 mL anhydrous 3A alcohol and increase vacuum to maximum.

Remove filter assembly from suction flask and transfer pad and residue to ashing dish. Repeat this filtration process for other samples at 2.25 min intervals. Dry pad and residue 2 h at 130 ± 2°C. Cool in desiccator and weigh. Ignite 30 min at 600 ± 15°C. Cool in desiccator and weigh.

% Crude fiber = (wt loss on ignition − wt loss of blank) × 100/sample wt.

Results and Discussion

The replacement of asbestos by ceramic fiber in the official method, **7.061-7.065** was made difficult by the different behavior of the 2 fibers. Because ceramic fiber does not disperse as readily as asbestos fiber, thicker pads of ceramic fiber were required to completely cover the screen of

Table 1. Crude fiber (%) determined by method 7.061–7.065 using ceramic (CF) or asbestos (AF) fiber

	CF		AF				
Sample	Mean	SD (*n*)	Mean	SD (*n*)	CF CV, %	AF CV, %	AF − CF
Pelleted alfalfa	31.91	0.62(8)	32.26	0.65(3)	1.96	2.02	+0.35
Pelleted alfalfa with molasses	27.58	(2)	27.83	(2)			+0.25
Dairy feed	6.91	(2)	7.14	(2)			+0.24
Horse feed	19.71	0.15(3)	20.34	(2)	0.74		+0.63
Rabbit feed	17.26	(2)	17.28	(2)			+0.02
Hen scratch	2.64	(2)	2.65	(2)			+0.01
Milking feed	4.06	(2)	4.23	(2)			+0.17
Turkey starter	3.35	(2)	3.45	(2)			+0.10
Pig starter	2.11	(2)	2.31	(2)			+0.21
Pet food (dry)	3.97	(2)	3.89	(2)			−0.08
Cattle supplement[a]	14.47	0.25(8)	15.04	(2)	1.74		+0.57
Broiler feed[a]	2.13	0.10(8)	2.36	(2)	4.80		+0.22
Beef finisher[a]	4.92	0.11(4)	5.12	0.18(4)	2.17	3.46	+0.21
Beef feed[a]	5.13	0.11(9)	5.18	0.12(4)	2.14	2.40	+0.05
Salmon fish food	3.00	0.13(3)	3.25	(2)	4.25		+0.24
40% Sow concentrate	6.71	0.09(3)	7.16	(2)	1.33		+0.45
Meat and bone meal	1.33	(2)	1.62	(2)			+0.30
Sunflower meal	18.64	0.27(3)	18.51	(2)			−0.14
Thrift pellets	20.67	(2)	20.49	(2)			−0.19
Dairy with bicarb.	7.72	(2)	7.20	(2)			−0.52
Sow gestation	7.68	(2)	7.81	(2)			+0.13
Sow mix	6.06	(2)	5.99	(2)			−0.07
Hog grower	5.05	(2)	5.00	(2)			−0.05
Hop pellets	22.96	(2)	22.13	(2)			−0.83
Blank (g)	0.0021	(2)					

[a] Compared with AAFCO values in Table 2.

Table 2. Comparison of crude fiber determination by method 7.061–7.065 using ceramic (CF) or asbestos (AF) fiber with AAFCO collaborative check sample data

			AF		
AAFCO No.	Sample (Table 1)		AAFCO mean	SD (*n*)	CF
8125	Cattle supplement	11	14.43	0.646(75)	14.47
8127	Broiler feed	12	2.31	0.216(67)	2.13
8128	Beef finisher	13	4.96	0.350(73)	4.92
8129	Beef feed	14	5.18	0.293	5.13

the filtering device. This thick pad tended to trap air and float away from the screen. Ceramic fiber in digestion beakers sank to the bottom instead of dispersing, and the trapped steam caused bumping. During filtering, this ceramic fiber remained in the bottom of the beaker until nearly all the solution was transferred. A layer of sample residue formed on the filter pad surface, partially plugging it.

These problems were greatly reduced by blending the fiber to disperse it and by modifying the filtering device to reduce the filtering area. Blending shortened the fiber length so that it remained dispersed in the digestion medium and poured more readily into the filtering device. Samples filtered rapidly because the residue remained dispersed and did not tend to form a mat. The sides of the filtering device were raised to allow faster and easier solution transfer. Reducing the filter area decreased by about 70% the amount of ceramic fiber needed to cover it, and increased the velocity through the filter pad so that the pad did not show much tendency to float. Bumping was dramatically reduced so that boiling chips were not needed in the digestion. These modifications resulted in a method which was significantly easier to use than the official method.

This method has the additional advantage that ceramic fiber does not require predigestion by acid and base followed by ignition, as prescribed for asbestos in the official method.

The developed method was tested by analyzing 24 feed samples representing a broad spectrum of feed types. The data were compared with results obtained when these same samples were analyzed by method **7.061-7.065** (Table 1).

Results ranged from 1.33 to 31.9% crude fiber by the ceramic fiber method. Both the ceramic fiber method and the official method appear to be of comparable precision. The ranges for coefficient of variation (CV), %, were 2.02-3.46 for the official method and 0.74-4.80 for the ceramic fiber method. The highest coefficient of variation (4.80%) occurred with a broiler feed which showed 2.13% crude fiber by the ceramic fiber method.

The 2 methods are well correlated ($r = 0.9994$), based on the mean values obtained from one or the other method applied to the various samples. Furthermore, linear regression yields a relationship of $Y = -0.106 + 1.001X$, where Y = ceramic fiber method and X = official method. The approximately 0.1% negative bias of the ceramic fiber method is about what could be predicted from examination of the data; this is small when compared with the uncertainty of either method (Table 1).

Table 2 compares results obtained using the ceramic fiber method against the grand average by the official method on several AFFCO Collaborative Check Samples. The ceramic fiber method yields data which compare favorably with results by the official method. The ceramic fiber method has the advantage of eliminating the use of asbestos, requires less preparation of the filter medium, and provides faster filtration.

REFERENCES

(1) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA

(2) *Asbestos: An Information Resource* (1978), R. J. Levine (Ed.), DHEW Publication Number (NIH) 78-1681

(3) Craddick, R. C., & Hatfield, L. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 1275-1276

Manual Salicylate-Hypochlorite Procedure for Determination of Ammonia in Kjeldahl Digests

HELEN E. FUKUMOTO and GEORGE W. CHANG[1]
University of California, Department of Nutritional Sciences, Berkeley, CA 94720

The determination of nitrogen in Kjeldahl digests of urine and feces has been simplified by using a manual spectrophotometric method with salicylate and hypochlorite. It neither involves the hazards of the phenol-hypochlorite method, nor requires an automated analyzer. We determined the conditions which minimize the need for precise timing and neutralization of acidic digests and obtained coefficients of variation of about 0.8%. Agreement between the spectrophotometric method and the conventional micro-Kjeldahl method was excellent; a correlation coefficient of 0.9992 and a coefficient of variation of the estimate of 2.1% were obtained. This method is well suited for a laboratory with a moderate volume of samples. We could process at least 200 digests per day, in contrast with the 60 per day which we had previously done by the Kjeldahl distillation and titration.

In 1883, Kjeldahl described a procedure for determinations of nitrogen in a variety of materials (1). This procedure involved destructive digestion of the sample in hot sulfuric acid followed by distillation and titration of the amounts produced. Numerous modifications of the method have appeared in the years since Kjeldahl's publication, and these are used for the analysis of a very wide spectrum of materials.

The major disadvantages of the Kjeldahl procedure are the time-consuming distillation and titration steps, limiting the number of samples which can be processed in a day. Attempts to solve these problems include the use of expensive automatic apparatus for the distillation and titration steps, or the use of an automated colorimetric assay for ammonia, which circumvents these steps (2). However, the purchase and the maintenance of the necessary equipment are beyond the means and needs of many laboratories.

Many popular colorimetric methods for ammonia determination involve the Berthelot reaction with phenol or α-chlorophenol and an active halogen to yield a colored product (3-11). Although simple, rapid, and precise, these methods have the disadvantage that phenol and chlorophenol are volatile enough to present a nuisance and a hazard for laboratory personnel.

Because of the hazardous nature of phenol, sodium salicylate has been used in its place. This reagent is much less volatile, but reaction with ammonia is slower and the color yield appears to be more sensitive to reaction conditions. Several procedures for the use of sodium salicylate have appeared in recent years for determination of nitrogen in serum, freshwater and seawater, and Kjeldahl digests (2, 12-19, and S. Greeley, University of California, Berkeley, personal communication, 1980). Three (of these procedures) use Kjeldahl digests; however, two are intended for automated apparatus and one requires manual pH adjustment of digests before analysis. These procedures require a wide variety of reagent concentrations and reaction conditions. In addition, reports of color stability conflict.

We felt that other laboratories whose volume of Kjeldahl nitrogen analyses does not justify purchase and maintenance of automated equipment might benefit from this rapid manual colorimetric method. Thus we have described our modification of other published salicylate procedures. Simple and convenient, it eliminates the necessity of adjusting the pH of the digests. With simple manual pipetting and dispensing devices, we now easily analyze over 200 digests per day, as contrasted to about 60 per day by the Kjeldahl method.

METHODS

Apparatus

(a) *Micro-Kjeldahl digestion rack.*—With 30 mL digestion flasks (ref. 2, sec. **47.022**).

(b) *Test tubes.*—16 × 125 mm screw-cap tubes.

(c) *Mixer.*—Vortex type (Van Waters & Rogers).

(d) *Spectrophotometer.*—Bausch & Lomb Spectronic 100 with continuous micro flow-through cell.

(e) *Water bath.*—45°C (Grant Instruments, UK).

[1] Address correspondence to this author.

Received August 24, 1981. Accepted February 23, 1982.

0004-5756/82/6505-1076-04$01.00

Reagents

Solutions were prepared volumetrically with deionized water. All chemicals were analytical reagent grade.

(a) *Hengar granules.*—Selenium-coated (Scientific Products, Menlo Park, CA).

(b) *Ammonium sulfate standard.*—Dry 2 h at 105°C. Dissolve 0.472 g ammonium sulfate in 50 mL water for working standard of 2 mg nitrogen/mL. Store at 4°C.

(c) *Sodium nitroprusside solution.*—Dissolve 78 mg sodium nitroprusside (NP) in 10 mL water. Store at 4°C in brown glass bottle. Stable ≥3 months.

(d) *Sodium salicylate–NP solution.*—Combine 125 g sodium salicylate and 10 mL sodium nitroprusside solution and dilute to 250 mL. Store in brown glass bottle at 4°C. Stable ≥3 months.

(e) *Tribasic sodium phosphate (TSP) solution.*—pH 12.5–12.7. Dissolve 110 g tribasic sodium phosphate dodecahydrate in water and dilute to 1 L. Measure pH.

(f) *Hypochlorite–TSP solution.*—Dilute 8 mL commercial bleach solution containing 5.25% active chloride (Clorox, or equivalent) to 50 mL with TSP solution. Use within 1 h .

Preparation of Sample

Accurately weigh or pipet sample or ammonium sulfate standard (0–3 mg nitrogen) into Kjeldahl flask. Add 2.0 mL H_2SO_4 and 1 Hengar granule. Place flask on digestion rack and set heater control to 4. When samples are boiling smoothly, turn control to 7. Digest sample until it is white or colorless. Turn off heater and let flask cool ca 5 min. Add 10 drops of 30% hydrogen peroxide. Turn control back to 7 and digest 15 min. Cool flask ca 10 min at room temperature or in refrigerator. Add 2–4 mL water, letting it rinse inner surfaces of flask. Cool flask again.

Nitrogen Determination

Dilute digested and cooled sample to 25 mL with water. Diluted samples should be assayed within 1 day, because longer storage, even at 4°C, causes loss of some ammonia. Undiluted digests can be stored overnight at 4°C; digests of ammonium sulfate standards are stable regardless of dilution.

Dispense 50 μL digest into a test tube. Into each tube, gently dispense 4.0 mL TSP solution. Turbulent mixing of alkaline TSP solution causes loss of ammonia. However, this mixture may sit at room temperature for up to 25 min without significant ammonia loss.

Add 0.4 mL salicylate–NP solution. Mix briefly but vigorously with vortex mixer. Add 0.55 mL hypochlorite–TSP solution. Mix with vortex mixer.

Incubate test tubes in 45°C water bath ca 20 min. Cool tubes in cold tap water bath. Read absorbance at 655 nm within 2 h.

For quality control, prepare standard curve every day and whenever new reagents are prepared.

Results and Discussion

The salicylate–hypochlorite method described here and a conventional micro-Kjeldahl method involving distillation and titration (F. Costa and R. Sandman, University of California, Berkeley, personal communication, 1974) were compared by using digests of homogenized fecal samples. The nitrogen content of 29 different homogenates ranged from 110 to 854 μg nitrogen/g homogenate digested, with a mean of 392. From the results of duplicate determinations, standard deviations were 10.0 and 13.5 for the salicylate and micro-Kjeldahl methods, respectively. Division of these values by the mean nitrogen content of the homogenates gave coefficients of variation of 0.82 and 0.94% for the respective methods. The correlation coefficient was 0.9992. The regression equation relating results of the salicylate method (X) to those of the micro-Kjeldahl (Y), in μg nitrogen/g homogenate digested, was $Y = 2.5 + 0.9917X$. The standard deviation of estimates of Kjeldahl nitrogen values based on salicylate results was 8.5 μg nitrogen/g homogenate. Division of this figure by the mean nitrogen content of the samples gives a coefficient of variation of the estimate of 2.1%. The minimum level of detection is approximately 0.06 μg and the linear range extends to at least 6 μg nitrogen per assay tube. This corresponds to 0.03 and 3.0 mg, respectively, per digestion with the dilution described in this paper.

It is important that the TSP solution have a pH of 12.5–12.7 to ensure that the final reaction mixture will have a pH of 12.0–12.4. Lower pH values result in reduced absorbances. Occasionally, bottles of TSP will not give solutions of the proper pH. These must not be used. The TSP solution described here will give an acceptable final pH even if there are reasonable differences in the H_2SO_4 remaining after the digestion. Differences of ±0.5 mL H_2SO_4 in the Kjeldahl flask before digestion do not significantly alter the standard curve or final nitrogen

values. Because it is not necessary to neutralize the sulfuric acid in a separate step, there is an important time-saving over some of the earlier procedures.

In the final reaction mixture, we found that the 4% sodium salicylate used by Searcy et al. (13) and the 0.0025% sodium nitroprusside used by Bower and Holm-Hansen (18) were optimal. Blank absorbance values were low (0.020–0.035). As the sodium salicylate–NP solution aged, the blank values increased.

Both sodium hypochlorite (2–5, 7, 9–11, 14, 17, 18) and sodium dichloroisocyanurate (6, 8, 12, 13, 16, 19) have served as sources of active chlorine. Even though the dichloroisocyanurate has been reported to be more stable in solution, we chose sodium hypochlorite because of its ready availability as a commercial laundry bleach. The 0.09% concentration of active chlorine in the final reaction mixture is similar to that used by Verdouw et al. (17) and Bower and Holm-Hansen (18). Increasing or decreasing the concentration by 50% had no effect on standard curves of absorbance vs ammonium nitrogen. Different bottles of bleach often contain different amounts of hypochlorite. Using a standard method (20), we found that one brand of bleach contained about 20% less hypochlorite than other brands did. However, this variation did not significantly affect final results. To minimize decomposition of bleach solutions, we store them at 4°C.

The rate of color development depends on temperature. At room temperature, maximum color development requires more than 20 min. However, at 45°C, color development is complete in 10 min. Furthermore, tubes could be left in the 45°C water bath for as long as 10–35 min without any significant effect on the final color yield. This makes the timing of this incubation step less critical and adds flexibility to the experiment.

Various sequences for adding reagents have been reported. It was advantageous to add TSP solution to the sample before any other reagents and thus prevent the precipitation of salicylic acid when the salicylate–NP solution is added. There is no loss of ammonia if the TSP is added gently. At this point, the tubes may be left at room temperature for at least 25 min, without loss of ammonia, thus eliminating the need to add the other reagents immediately. However, the salicylate–NP and hypochlorite solutions should be added in quick succession.

An apparent extinction coefficient for the product(s) of the reaction can be calculated from standard curves. Seven such curves yielded an extinction coefficient of 19 290 ± 40 (standard error of the mean) mole $^{-1}$L cm^{-1}. The absorbances of standards and unknowns drop by about 0.3% per hour for the first few hours after color development. In 24 h, they diminish about 3%. Even though analyzing standards along with unknowns should compensate for such changes, it is best to measure absorbances within 1–2 h.

Ion interference apparently presents no problem with the salicylate–hypochlorite method (16, 17). We were concerned that selenium from the catalyst granules used in the digestion might interfere. However, the use of up to 3 Hengar granules did not affect the final absorbance of standards.

Dispensing devices make analyses much more efficient. We use a Pipetman variable-volume dispenser (West Coast Scientific, Oakland, CA) for transferring 50 μL portions of diluted digest, and Repipets (Labindustries, Berkeley, CA) to add TSP buffer, salicylate–NP solution, and hypochlorite–TSP solution. Many of our nitrogen analyses involve homogenates of feces. In order to obtain uniform samples, we stir fecal homogenates with a magnetic stirring bar and sample them with a Pasteur pipet with the tip broken off to provide a 3–4 mm orifice.

Acknowledgment

This work was supported by Grant PFR-79-19105 from the National Science Foundation. We thank Marc Schelstraete for his critical evaluation of the method and his many suggestions.

References

(1) Kjeldahl, J. (1883) *Z. Anal. Chem.* **22,** 366–382
(2) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA., sec. **7.021–7.031**
(3) Horn, D. B., & Squire, C. R. (1967) *Clin. Chim. Acta* **17,** 99–105
(4) Gehrke, C. W., Kaiser, F. E., & Ussary, J. P. (1968) *J. Assoc. Off. Anal. Chem.* **51,** 200–211
(5) Harwood, J. E., & Kuhn, A. L. (1970) *Water Research* **4,** 805–811
(6) Yamaguchi, R., Suzuki, H., & Hirashima, H. (1980) *Chem. Pharm. Bull.* **18,** 1866–1871
(7) Shahinian, A. H., & Reinhold, J. G. (1971) *Clin. Chem.* **17,** 1077–1080
(8) Liddicoat, M. I., Tibbitts, S., & Butler, E. I. (1975) *Limnol. Oceanogr.* **20,** 131–132
(9) Scheiner, D. (1976) *Water Res.* **10,** 31–36
(10) Patton, C. J., & Crouch, S. R. (1977) *Anal. Chem.* **49,** 464–469
(11) McCracken, M. S., & Malmstadt, H. V. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 23–28
(12) Reardon, J., Foreman, J. A., & Searcy, R. L. (1966)

Clin. Chim. Acta **14,** 403–405
(13) Searcy, R. L., Reardon, J. E., & Foreman, J. A. (1967) *Am. J. Med. Technol.* **33,** 15–20
(14) Wall, L. L., Sr, & Gehrke, C. W. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 1221–1226
(15) Bietz, J. A. (1974) *Anal. Chem.* **46,** 1617–1618
(16) Pym, R. V. E., & Milham, P. J. (1978) *Anal. Chem.* **48,** 1413–1415
(17) Verdouw, H., Van Echteld, C. J. A., & Dekkers, E. M. J. (1978) *Water Res.* **12,** 399–402
(18) Bower, C. E., & Holm-Hansen, T. (1980) *Can. J. Fish. Aquat. Sci.* **37,** 794–798
(19) Crooke, W. M., & Simpson, W. E. (1971) *J. Sci. Food Agric.* **22,** 9–10
(20) *Standard Methods for the Examination of Water and Wastewater* (1971) 13th Ed., American Public Health Association, Washington, DC, pp. 107–112

COLOR ADDITIVES

High Performance Liquid Chromatographic Determination of Subsidiary Colors in FD&C Red No. 3

ROBERT J. CALVEY and ALLEN L. GOLDBERG
Food and Drug Administration, Division of Color Technology, Washington, DC 20204

Two rapid, sensitive, reproducible methods that use a Zorbax C-8 reverse phase column and high performance liquid chromatography are described for the determination of the subsidiary colors in FD&C Red No. 3. With the first method, 8 subsidiary colors (fluorescein, 2'-iodofluorescein, 4'-iodofluorescein, 2',5'-diiodofluorescein, 2',7'-diiodofluorescein, 4',-5'-diiodofluorescein, 2',4',7'-triiodofluorescein, and 2',4',5'-triiodofluorescein) are eluted in a reproducible pattern by increasing the organic nature of a buffered mobile phase. Method 1 is capable of quantitating all the subsidiary colors except 4'-iodofluorescein and 4',5'-diiodofluorescein. If these 2 subsidiary colors are seen, the sample must be run again by method 2, which uses a different program and solvent system to quantitate them. The average recoveries for the 6 subsidiary colors quantitatively determined in FD&C Red No. 3 by method 1 ranged from 96 to 98%. The average recoveries for 4'-iodofluorescein and 4',5'-diiodofluorescein, quantitatively determined in FD&C Red No. 3 by method 2, were 101 and 103%, respectively. The amounts of the 6 subsidiary colors recovered by method 1 were 0.04-7.4% by weight of the total sample. The amounts of the 2 subsidiary colors recovered by method 2 were 0.13-2.6% by weight of the total sample.

Two rapid, sensitive, reproducible procedures that use high performance liquid chromatography (HPLC) have been developed for the determination of the subsidiary colors in FD&C Red No. 3.

FD&C Red No. 3 (Colour Index No. 45430) is a synthetic color additive that is prepared by condensation of resorcinol with phthalic anhydride to produce fluorescein (Figure 1), which in turn is iodinated and treated with NaOH to produce FD&C Red No. 3 (Figure 2). During the iodination of fluorescein to make FD&C Red No. 3, many lower iodinated fluoresceins (subsidiary colors) may be produced.

Every batch of certifiable FD&C Red No. 3 that is manufactured must first be chemically analyzed and approved by the Certification Branch, Division of Color Technology, Food and Drug Administration before it can be used in a food, drug, or cosmetic in the United States. The Code of Federal Regulations (CFR) (1) limits the amounts of the subsidiary colors. Monoiodofluoresceins are limited to not more than 1.0%, and other lower iodinated fluoresceins to not more than 9.0% by weight of FD&C Red No. 3. There is no limit specifically expressed in the regulations for fluorescein. The CFR requires, however, that limits for such impurities be set in accordance with good manufacturing practice.

At the present time in the Certification Branch, the subsidiary colors in FD&C Red No. 3 are being determined by a thin layer chromatographic (TLC) method. This method, which takes about 2½ h to complete, does not separate all of the subsidiary colors from each other or from the main color band.

Both in method 1 and in method 2, the subsidiary colors in FD&C Red No. 3 are separated by the use of a buffered mobile phase. A sample of dye is dissolved in water and then is injected onto the column. As the solvent passes through the Zorbax C-8 column, the organic nature of the mobile phase is varied by a gradient elution system. As the organic nature of the mobile phase increases, the subsidiary colors are selectively released and elute in a reproducible pattern. In method 1, fluorescein and the mono- and diiodinated fluoresceins are quantitated from their visible absorbances at 436 nm. The triiodinated fluoresceins are quantitated from their visible absorbances at 546 nm. Method 2 is used when 4'-iodofluorescein and/or 4',5'-diiodofluorescein are detected by method 1 because these 2 subsidiary colors co-elute in method 1. Both of these subsidiary colors are quantitated from visible absorbances at 436 nm.

METHOD

Reagents

(a) *Water.*—Deionized, distilled, obtained from Milli-Q Water Purification System (Millipore Corp., Bedford, MA 01730), or equivalent.

Received July 31, 1981. Accepted February 4, 1982.
This paper was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

0004-5756/82/6505-1080-06$01.00

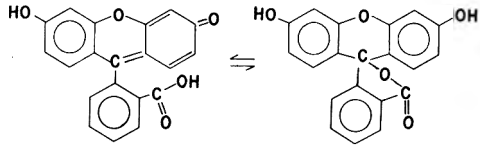


Figure 1. Fluorescein.

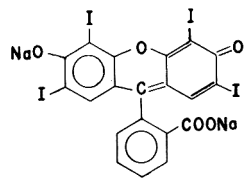


Figure 2. FD&C Red No. 3.

(b) *Eluants.*—Method 1 uses the following solvents: primary solvent A, 0.1M ammonium acetate; secondary solvent B, methanol. Method 2 uses the following solvents: primary solvent A, 2% (v/v) glacial acetic acid in water, secondary solvent B, acetonitrile.

(c) *Stock solutions for calibrations.*—For each of the 2 triiodofluoresceins, weigh 140–200 mg subsidiary color, add 3 drops concentrated NH_4OH to the color, dissolve in water, transfer to 100 mL volumetric flask, and dilute to volume with water. For each of the 3 diiodofluoresceins, weigh 50–110 mg subsidiary color, add 3 drops concentrated NH_4OH to the color, dissolve in water, transfer to 100 mL volumetric flask, and dilute to volume with water. For fluorescein and for each of the 2 monoiodofluoresceins, weigh 15–30 mg subsidiary color, add 3 drops concentrated NH_4OH to the color, dissolve in water, transfer to 100 mL volumetric flask, and dilute to volume with water. Determine exact concentration of each stock solution from the visible spectra of aliquots diluted to volume with water in 100 mL volumetric flask after addition of 2 drops of concentrated NH_4OH. The concentration of each solution that is spectrophotometrically measured should be ca 10 mg/L. Approximate absorptivities, *a* (L/mg/cm) (C. Graichen, Division of Color Technology, 1963), are 2′-iodofluorescein (2-Iodo), 0.193 at 500 nm; 4′-iodofluorescein (4-Iodo), 0.154 at 497 nm; 2′,5′-diiodofluorescein (2,5-Diiodo), 0.145 at 509 nm; 2′,7′-diiodofluorescein (2,7-Diiodo), 0.179 at 511 nm; 4′,5′-diiodofluorescein (4,5-Diiodo), 0.122 at 507 nm; 2′,4′,5′-triiodofluorescein (2,4,5-Triiodo), 0.116 at 516 nm; 2′,4′,7′-triiodofluorescein (2,4,7-Triiodo), 0.140 at 517 nm; and fluorescein (Y-7), 0.247 at 489 nm. Store all stock solutions in the dark when not in use.

Apparatus

(a) *Liquid chromatograph.*—With gradient elution capability. Altex Model 420 equipped with microprocessor and 2 Model 110-A pumps (Altex Scientific Inc., Berkeley, CA 94710), or equivalent. Operating conditions: chart speed 0.2 in./min; flow rate 1 mL/min; column temperature ambient. The gradient program for method 1 uses program 1; the individual steps are as follows: step 1, start auto-injector and then start step 2 after 0.99 min; step 2, 45 to 66% B in 20 min; step 3, 66 to 100% B in 0 min; step 4, hold at 100% B for 4 min; step 5, 100 to 45% B in 1 min; step 6, hold at 45% B for 4 min; step 7, start step 1. The gradient program for method 2 uses program 2; the individual steps are as follows: step 1, start auto-injector and then start step 2 after 0.99 min; step 2, hold at 43% B for 15 min; step 3, 43 to 100% B in 3 min; step 4, hold at 100% B for 4 min; step 5, 100 to 43% B in 2 min; step 6, hold at 43% B for 5 min; step 7, start step 1. The individual steps listed for both gradient programs are entered in the microprocessor. When the auto-injector is started, it first flushes the 20 μL loop with water, next the loop is flushed with the FD&C Red No. 3 sample, and then the sample is injected onto the column; these operations are performed in a total time of 0.99 min by this auto-injector. If an auto-injector is unavailable, inject samples manually and start step 2 immediately after injection.

(b) *Detector.*—Waters Model 440 dual wavelength detector (Waters Associates, Inc., Milford, MA 01757), or equivalent, set at the following wavelengths: 546 nm (method 1, attenuation set at 0.2 AUFS; method 2, attenuation set at 0.1 AUFS) and 436 nm (method 1, attenuation set at 0.1 AUFS; method 2, attenuation set at 0.02 AUFS).

(c) *2-Pen recorder.*—Soltec Model 3314 (Soltec Corp., Sun Valley, CA 91352), or equivalent.

(d) *Injector.*—Micromeritics Model 725 auto-injector equipped with 20 μL sampling loop (Micromeritics Instrument Corp., Norcross, GA 30093), or equivalent.

(e) *Chromatographic column.*—DuPont Zorbax C-8, 25 cm × 4.6 mm id (Cat. No. 850952-706, DuPont Instruments, Wilmington, DE 19898), or equivalent.

(f) *Spectrophotometer.*—Visible range.

Calibration

Using method 1, construct calibration plots for Y-7, 2-Iodo, 2,5-Diiodo, 2,7-Diiodo, 2,4,5-Triiodo, and 2,4,7-Triiodo from the chromatographic analysis of standard solutions prepared as follows: Dissolve 200 mg FD&C Red No. 3 (shown by previous analysis to be free of all subsidiary colors) in water and transfer to 100 mL volumetric flask. Add appropriate aliquots (0.5–10 mL) of stock solutions of the 6 subsidiary colors to the volumetric flask and bring to volume with water. Analyze 6 calibration solutions containing fairly evenly spaced concentrations of each subsidiary color. For each subsidiary color in calibration solution, calculate C, % by weight relative to amount of FD&C Red No. 3 in calibration solution:

$$C = V \times C' \times 100\ (\%) \times (1/200\ \text{mg})$$

where V = volume of stock solution aliquot (mL), and C' = concentration of stock solution (mg/mL) determined spectrophotometrically.

Using method 2, construct calibration plots for 4-Iodo and 4,5-Diiodo from the chromatographic analysis of standard solutions prepared as follows: Dissolve 200 mg FD&C Red No. 3 (shown by previous analysis to be free of all subsidiary colors) in water and transfer to 100 mL volumetric flask. Add appropriate aliquots (1–10 mL) of stock solutions of the 2 subsidiary colors to volumetric flask and dilute to volume with water. Analyze 5 calibration solutions containing fairly evenly spaced concentrations of the 2 subsidiary colors. For the 2 subsidiary colors in the calibration solution, calculate C as stated previously.

When using method 1 or 2 with auto-injector, position calibration solutions in autosampler as follows: position 1, blank vial (containing water); position 2, rinse vial containing water; position 3, calibration solution; all other even-numbered positions contain rinse vials and odd-numbered positions contain FD&C Red No. 3 samples.

Use peak height to develop calibration plots for all 8 subsidiary colors. Mathematically choose best fitting straight line for calibration data by method of least squares. For each of the 8 subsidiary colors, calculate regression line, $y = bx + a$, by using the following equations:

$$b = [\Sigma(x - \bar{x})(y - \bar{y})]/[\Sigma(x - \bar{x})^2]$$

$$= [\Sigma(xy) - [(\Sigma x \Sigma y)/n]]/[\Sigma x^2 - [(\Sigma x)^2/n]]$$

$$a = \bar{y} - b\bar{x}$$

where x = concentration of subsidiary color in calibration standard; y = peak height response to calibration standard for each subsidiary color; n = number of determinations; b = slope of regression line; and a = y intercept of line.

Determine linear correlation between peak height and concentration of standards by calculating correlation coefficient, r:

$$r = [\Sigma(x - \bar{x})(y - \bar{y})]/\sqrt{[\Sigma(x - \bar{x})^2][\Sigma(y - \bar{y})^2]}$$

Determination

Dissolve 200 mg FD&C Red No. 3 in ca 50 mL water. Transfer quantitatively to 100 mL volumetric flask. Dilute to volume with water and mix well. Fill auto-injector vial in position 5 with this solution and place in auto-injector. After all vials are in place, start gradient program 1.

From regression line equation, $y = bx + a$, calculate x, the % of each subsidiary color in the FD&C Red No. 3 sample, by substituting value of y (peak height of subsidiary color) and solving for x.

If chromatogram of FD&C Red No. 3 sample indicates presence of 4-Iodo or 4,5-Diiodo (Figure 3, peak 2), sample must be re-analyzed using method 2. Auto-injector and sample preparations are the same as stated above for method 1. Figure 4 shows order of elution of subsidiary colors with method 2. Calculate % of each of the 2 subsidiary colors as stated previously.

Results and Discussion

For the development of method 1, we used concentrations of the 6 quantitatively determined subsidiary colors in 11 calibration solutions that covered the following ranges, expressed as % by weight of FD&C Red No. 3: Y-7, 0.08–0.58%; 2-Iodo, 0.13–0.90%; 2,5-Diiodo, 0.37–3.7%; 2,7-Diiodo, 0.51–5.1%; 2,4,7-Triiodo, 0.87–7.6%; and 2,4,5-Triiodo, 0.93–7.6%. For method 2, we used concentrations of the 2 quantitatively determined subsidiary colors in 5 calibration solutions that covered the following ranges, expressed as % by weight of FD&C Red No. 3: 4-Iodo, 0.13–0.65%; and 4,5-Diiodo, 0.51–2.6%. We have stored both stock and calibration solutions for 6 months in the dark without noticeable degradation.

The following limits of detection were calculated for method 1 according to the technique of statistical analysis described by Bailey et al. (2): Y-7, 0.04%; 2-Iodo, 0.06%; 2,5-Diiodo, 0.3%; 2,7-Diiodo, 0.5%; 2,4,7-Triiodo, 0.4%; and 2,4,5-Tri-

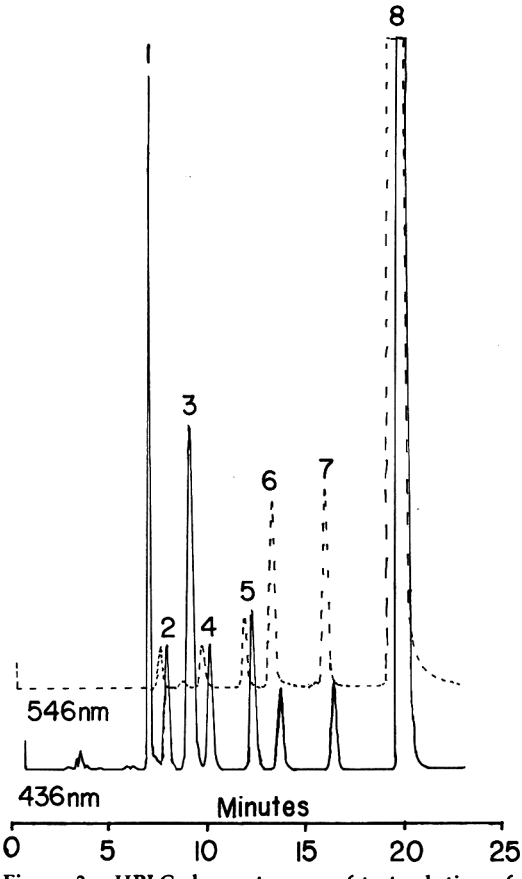


Figure 3. HPLC chromatogram of test solution of FD&C Red No. 3 (peak 8). 1 = Y-7; 2 = 4-Iodo and 4,5-Diiodo; 3 = 2-Iodo; 4 = 2,5-Diiodo; 5 = 2,7-Diiodo; 6 = 2,4,5-Triiodo; 7 = 2,4,7-Triiodo. Abbreviations are defined under *Reagents*, (c) *Stock solutions for calibrations.*

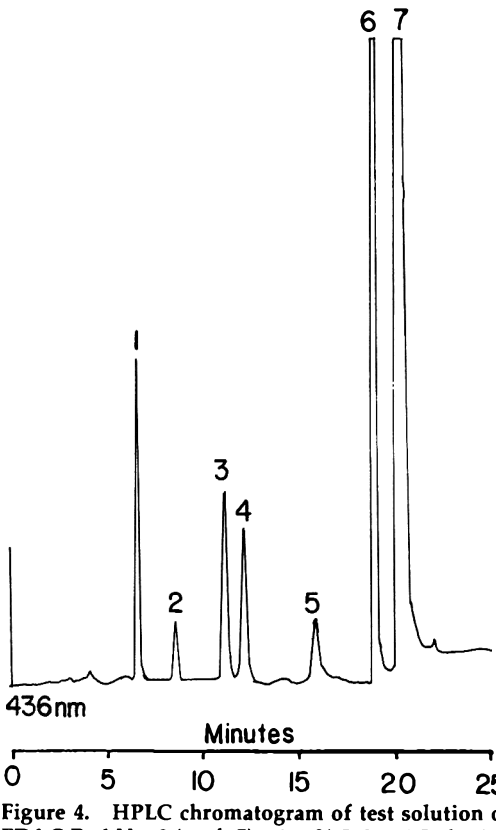


Figure 4. HPLC chromatogram of test solution of FD&C Red No. 3 (peak 7). 1 = Y-7; 2 = 4-Iodo; 3 = 2-Iodo; 4 = 4,5-Diiodo; 5 = 2,5-Diiodo; 6 = 2,7-Diiodo and 2,4,5-Triiodo; 7 = 2,4,7-Triiodo and FD&C Red No. 3. Abbreviations are defined under *Reagents*, (c) *Stock solutions for calibrations.*

iodo, 0.7%. The limits of detection for method 2 were as follows: 4-Iodo, 0.11%; and 4,5-Diiodo, 0.3%.

Recovery studies with method 1 gave the following recovery ranges and averages for the subsidiary colors: Y-7, 93.9–102%, 96%; 2-Iodo, 94.1–100%, 98%; 2,5-Diiodo, 87.8–100%, 96%; 2,7-Diiodo, 84.3–106%, 98%; 2,4,7-Triiodo, 86.7–104%, 98%; and 2,4,5-Triiodo, 86.7–104%, 97%. The recovery ranges and averages for method 2 are as follows: 4-Iodo, 90.4–108%, 101%; and 4,5-Diiodo, 90–114%, 103%. Recovery data for the individual determinations are given in Tables 1 and 2. The paired-difference 99% confidence intervals all included zero.

The calibration and recovery data were calculated twice—once using the manual measurement of peak height and once using the manual measurement of peak area. As found in earlier work (3), we obtained much better results by using peak height (4) because of the great variability in the measurement of the small peak width at half peak height. It appears that 0.1 mm is the minimum error inherent in the measurement of either peak height or peak width. Thus the measurement of a small peak at half peak height, which is needed for the area calculation, introduces a larger percentage error than the measurement of peak height, which is usually much larger than peak width. Although the use of an electronic integrator would minimize the greater error in the peak area measurements, peak area rather than peak height measurements are needed in situations involving peaks of greatly different widths.

Using method 1, we analyzed 36 different FD&C Red No. 3 samples. 2,4,5-Triiodo and 2,4,7-Triiodo were the only subsidiary colors detected in these samples. For this reason, we suggest that standards containing only these 2 subsidiary colors be run daily. If other subsidi-

Table 1. Recovery data and paired-difference 99% confidence interval results for method 1

	Subsidiary color, %			Subsidiary color, %		
Detn	Added	Found	Rec.	Added	Found	Rec.
	Fluorescein			2′-Iodofluorescein		
1	0.58	0.56	96.6	0.13	0.13	100
2	0.083	0.085	102	1.2	1.2	100
3	0.25	0.24	96.0	0.26	0.25	96.2
4	0.041	0.039	95.1	0.38	0.36	94.7
5	0.17	0.16	94.1	0.51	0.50	98.0
6	0.33	0.32	97.0	0.64	0.63	98.4
7	0.41	0.40	97.6	0.90	0.88	97.8
8	0.25	0.24	96.0	0.26	0.25	96.2
9	0.17	0.16	94.1	0.51	0.48	94.1
10	0.33	0.31	93.9	0.60	0.60	100
Av. recovery			96			98
Paired-difference 99% confidence interval:						
		−0.01 ± 0.01			−0.01 ± 0.01	
	2′,5′-Diiodofluorescein			2′,7′-Diiodofluorescein		
1	0.37	0.33	89.2	1.0	0.92	92.0
2	0.74	0.65	87.8	0.51	0.43	84.3
3	1.1	1.1	100	1.5	1.5	100
4	2.2	2.1	95.5	3.1	3.0	96.8
5	1.9	1.8	94.7	2.6	2.5	96.2
6	3.7	3.6	97.3	5.1	5.1	100
7	1.5	1.5	100	2.0	2.1	105
8	2.2	2.2	100	3.1	3.1	100
9	3.0	3.0	100	4.1	4.2	102
10	2.6	2.6	100	3.6	3.8	106
Av. recovery			96			98
Paired-difference 99% confidence interval:						
		−0.04 ± 0.05			−0.0 ± 0.1	
	2′,4′,5′-Triiodofluorescein			2′,4′,7′-Triiodofluorescein		
1	1.5	1.3	86.7	1.5	1.3	86.7
2	0.74	0.66	89.2	0.74	0.69	93.2
3	3.7	3.5	94.6	3.7	3.6	97.3
4	7.4	7.1	95.9	7.4	7.1	95.9
5	2.2	2.1	95.5	2.2	2.2	100
6	4.5	4.4	97.8	4.5	4.5	100
7	3.0	3.0	100	3.0	3.0	100
8	4.5	4.5	100	5.9	6.0	102
9	5.9	6.0	102	5.2	5.4	104
10	5.2	5.4	104	NA[a]	NA	NA
Av. recovery			97			98
Paired-difference 99% confidence interval:						
		−0.1 ± 0.2			0.0 ± 0.2	

[a] NA = not added.

Table 2. Recovery data and paired-difference 99% confidence interval results for method 2

	4′-Iodofluorescein, %			4′,5′-Diiodofluorescein, %		
Detn	Added	Found	Rec.	Added	Found	Rec.
1	0.13	0.14	108	2.6	2.6	100
2	0.39	0.41	105	1.0	1.0	100
3	0.52	0.47	90.4	2.0	1.8	90.0
4	0.26	0.26	100	1.5	1.7	113
5	0.65	0.65	100	0.51	0.58	114
Av. recovery			101			103
Paired-difference 99% confidence interval:						
		0.0 ± 0.06			0.0 ± 0.3	

aries are seen in a sample of FD&C Red No. 3, then a standard containing those subsidiary colors should be run.

Each set of acceptable calibration data is added to the preceding data and a new regression line and coefficient of correlation are calculated. The correlation coefficient should be between 0.95 and 1.00. New calibration plots should be constructed if the correlation coefficient is less than 0.95.

REFERENCES

(1) *Code of Federal Regulations* (1981) Title 21, U.S. Government Printing Office, Washington, DC, sec. **74.303(b)**

(2) Bailey, C. J., Cox, E. A., & Springer, J. A. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1404–1414

(3) Calvey, R. J., Goldberg, A. L., & Madigan, E. A. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 665–669

(4) Snyder, L. R., & Kirkland, J. J. (1974) *Introduction to Modern Liquid Chromatography*, John Wiley & Sons, New York, NY, pp. 434–435

EXTRANEOUS MATERIALS

Extraction of Light Filth from Rice Flours, Extruded Rice Products, and Rice Paper: Collaborative Study

RUSSELL G. DENT
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Collaborators: J. R. Bryce; B. C. Cox; D. D. Hughes; J. T. Karpus; J. Nagy; D. J. Vail

Two new methods were developed for the extraction of rodent hairs and insect fragments from rice products: one for rice flour and one for extruded rice products and rice paper. A 100 g sample of rice flour was extracted with mineral oil-40% isopropanol, followed by a water phase as needed for additional cycles. For extruded rice products and rice paper, a 225 g sample of each was initially extracted as above, followed by a single extraction with mineral oil-20% isopropanol. Both methods used an acid hydrolysis pretreatment followed by wet sieving and a percolator extraction. Average rodent hair recoveries were 77.8% for rice flour and 82.2% for extruded rice products and rice paper. Average insect fragment recoveries were 89.6% for rice flour and 91.9% for extruded rice products and rice paper. Both methods were adopted official first action.

Two methods were developed for rice products: one for flour and another for extruded rice products and rice paper. The methods differ only in amounts and concentrations of reagents and certain time frames, which are needed to accommodate the different sample sizes for flour (100 g) and extruded products and paper (225 g). Although all of these products are derived from milled rice, their analytical characteristics vary. Two kinds of rice flour, plain and sweet, are commercially available. Both are made from milled rice, i.e., rice with husk and bran removed. Because sweet rice flour contains very little amylose, it is difficult to hydrolyze. Extruded rice products and rice paper, which are made from cooked rice flour and contain much more amylose, respond well to acid hydrolysis pretreatment.

Received August 26, 1981. Accepted October 26, 1981.
This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.
The recommendation of the Associate Reference was approved by the General Referee and Committee F and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) **65**, 387.

Light Filth in Rice Flours (Powders), Extruded Rice Products, and Rice Paper

Official First Action

44.C04 ***Sample Preparation***

(**a**) *Rice flours (powders).*—Preheat hot plate to max. heat. Add mag. stirring bar to 2 L beaker, and tare. Add 100 g sample. With forceful stream, add ca 100 mL hot tap H_2O. Add 75 mL HCl and fill to 800 mL mark with hot tap H_2O. Place hot mixt. on hot plate and, with mag. stirring, bring mixt. to vigorous boil. Boil 5 min. In small increments, transfer hot mixt. to No. 230 sieve. Reserve 2 L beaker. Wash residue with forceful stream of hot tap H_2O until foaming has subsided and H_2O is clear. Transfer residue to reserved 2 L beaker with 40% isopropanol. Add mag. stirring bar. Fill with 40% isopropanol to 800 mL mark. With mag. stirring, bring to boil on hot plate. Add 95 mL mineral oil, **44.003(y)**, and boil and stir 3 min.

(**b**) *Extruded rice products and rice paper.*—Preheat hot plate to max. heat. Add mag. stirring bar to a 2 L beaker, and tare. Break up 225 g extruded product or paper while weighing into tared beaker. Add ca 1450 mL hot tap H_2O mixed with 150 mL HCl. Hand-stir with glass rod while heating to vigorous boil. When product is fluid enough, use mag. stirring. Boil 10 min. In small increments, transfer hot mixt. to No. 230 sieve. Reserve 2 L beaker. Wash residue with forceful stream of hot tap H_2O until foaming has subsided and H_2O is clear. Transfer residue to reserved 2 L beaker with 40% isopropanol. Add mag. stirring bar. Fill with 40% isopropanol to 800 mL mark. With mag. stirring, bring to boil on hot plate. Add 95 mL mineral oil, **44.003(y)**, and boil and stir 3 min.

44.C05 ***Filth Analysis***

(**a**) *Rice flours (powders).*—Clamp off rubber hose on percolator, **44.002(i)**(2). Add 300 mL

0004-5756/82/6505-1086-03$01.00

40% isopropanol. Transfer hot sample mixt. from above to percolator. Rinse 2 L beaker with 40% isopropanol and pour rinse into percolator. With same beaker, add enough 40% isopropanol (ca 1 L) to fill percolator within 3 cm of top. Let stand 5 min and drain contents to 5 cm from bottom of oil layer. Repeat fill and drain steps at 2 min intervals with hot tap H_2O until aq. phase is clear. Drain as above. Drain oil layer into 1 L beaker. Rinse percolator sides with several alternate washes of H_2O, 40% isopropanol, and isopropanol, collecting rinsings in same 1 L beaker. A 1% sodium lauryl sulfate soln may also be used if needed for final rinse. Filter onto ruled filter paper and examine microscopically at ca 30×.

(b) *Extruded rice products and rice paper.*—Proceed as in (a), except for second cycling. Refill percolator with 20% isopropanol. Let stand addnl 5 min. Drain oil layer into 1 L beaker and rinse percolator as above.

Collaborative Study

The statistical design was the same for both methods. Each of the collaborators examined 3 subsamples of plain rice flour and 3 of sweet rice flour by 1 method and 3 subsamples of extruded rice products and 3 of rice paper by the other proposed method.

The spike material for each subsample contained 20 elytral fragments ca 0.5 mm sq. and 20 rodent hairs ca 2 mm long. The collaborators were instructed to report their recoveries of spike material and to return extraction plates to the Associate Referee for confirmation of the count.

Results and Discussion

Average recoveries for the rice flour method were 89.6% for insect fragments and 77.8% for rodent hairs (Table 1). Average recoveries for the extruded rice products and rice paper were 91.9% for insect fragments and 82.2% for rodent hairs (Table 2).

All collaborators agreed that both methods were easy to perform. Two collaborators had difficulties with the initial pretreatment of rice flour and the cleanup procedures of the extraction. These problems were corrected by clarifying the procedure for adding acid and water in the pretreatment, specifying "several" alternate washes of water and isopropanol, and including an additional final wash of 1% sodium lauryl sulfate if needed. Corresponding changes were made in the applicable sections of the extruded rice products and rice paper method.

Analytical time per subsample averaged 1.5–2.0 h for both methods. Although most analysts examined subsamples in pairs, 2 analyzed all 6 of the subsamples for a given method at one

Table 1. Collaborative results for the recovery [a] of insect fragments and rodent hairs from plain rice flour [b] and sweet rice flour [c] by the proposed method

	Insect fragments (20 added)						Rodent hairs (20 added)					
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
A	12	11	19	16	15	16	12	13	15	9	10	13
B	16	19	18	19	19	20	19	16	17	15	19	19
C	18	20	19	19	16	18	9	18	12	16	11	16
D	20	16	20	18	20	18	15	13	19	14	13	18
E	18	20	18	20	17	19	18	16	17	19	16	17
F	16	19	18	19	19	21(20)	15	18	20	18	22(20)	15
Av. No.					17.94(17.92)						15.61(15.56)	
Av., %					89.72(89.59)						78.06(77.78)	
SD, %												
Repeatability					1.79(1.75)						2.44(2.36)	
Reproducibility					2.24(2.20)						3.27(3.17)	
CV, %												
Repeatability					9.95(9.77)						15.65(15.14)	
Reproducibility					12.46(12.29)						20.95(20.37)	

[a] Associate Referee counts in parentheses.
[b] Subsamples 1–3, plain rice flour.
[c] Subsamples 4–6, sweet rice flour.

Table 2. Collaborative results for the recovery [a] of insect fragments and rodent hairs from extruded rice products [b] and rice paper [c] by the proposed method

	Insect fragments (20 added)						Rodent hairs (20 added)					
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
A	16	20	[d]	20	19	19	11	19	[d]	19	17	18
B	20	19	20	18	17	16	19	17	17	17	17	15
C	18	22(20)	15	24(20)	14	19	16	9	12	22(20)	18	18
D	15	16	20	17	17	15	17	19	15	16	11	12
E	20	20	19	19	18	[d]	20	18	18	21(20)	21(20)	[d]
F	22(20)	20	20	19	21(20)	24(20)	15	13	21(20)	12	14	23(20)
Av. No.					18.77(18.38)						16.68(16.44)	
Av., %					93.85(91.91)						83.40(82.20)	
SD, %												
Repeatability					2.20(1.67)						3.38(3.00)	
Reproducibility					2.45(1.89)						3.42(3.10)	
CV, %												
Repeatability					11.73(9.11)						20.27(18.27)	
Reproducibility					13.04(10.30)						20.51(18.82)	

[a] Associate Referee counts in parentheses.
[b] Subsamples 1–3, extruded rice products.
[c] Subsamples 4–6, rice paper.
[d] Lost in analysis.

time. Counting time and ease of reading papers varied with the experience of the analyst and the amount of plant material on the filth papers, which ranged from very clean to heavy. The heavy papers were caused by insufficient digestion; therefore, instructions for preheating the hot plate to maximum heat were given at the very beginning of each method and a vigorous boil was specified.

Recommendation

It is recommended that the proposed methods for the extraction of light filth from plain rice flour, sweet rice flour, extruded rice products, and rice paper be adopted official first action, based on the results of this collaborative study.

Acknowledgments

The author thanks the following collaborators, all of the Food and Drug Administration: J. R. Bryce, Los Angeles, CA; B. C. Cox, Dallas, TX; D. D. Hughes, Kansas City, MO; J. T. Karpus, Chicago, IL; J. Nagy, New York, NY; D. J. Vail, Atlanta, GA. The author also thanks the following, all of the Food and Drug Administration, Bureau of Foods, Washington, DC: J. S. Gecan and J. L. Boese for reviewing the manuscript and F. McClure for statistical analysis.

Defatting Technique for Two Ground Spices Using Simple Reflux Apparatus: Collaborative Study

RUSSELL G. DENT
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Collaborators: R. B. Bradicich; G. Dzidowski; L. Glaze; J. Quaife

A solvent defatting technique was tested for 2 spices which require the same extraction process but different defatting agents. For turmeric, which uses isopropanol as the defatting agent, recoveries of rodent hair and insect fragments were not significantly different from those in the original collaborative study, and this technique is recommended as an alternative to AOAC method 44.122(a). However, for oregano, which uses chloroform as the defatting agent, recoveries of insect fragments were significantly lower by the reflux method. An alternative chloroform pretreatment for oregano, therefore, is not recommended for AOAC 44.122(b). It is suggested that reflux action defatting techniques be considered during the developmental stages of new methods. The technique has been adopted official first action for turmeric.

Many AOAC methods for spices require 3 or more open hot solvent defatting or wash steps. This simple reflux, or "solvent saver," technique attempts to reduce the amount of solvent required and confine solvent vapors to the reflux apparatus. AOAC pretreatment method **44.122(a)** requires 3 portions of isopropanol and a 10 min boil for each change; pretreatment method **44.122(b)** requires 3 changes of chloroform, a 10 min boil for each change, and a short wash with isopropanol after the last change. A beaker is used for all of the boiling steps in both methods to permit release of the defatting solvent and plant volatiles into the atmosphere.

The solvent saver apparatus (Figure 1) consists of support stands with clamped 1 L round-bottom flasks which are stoppered with 2-hole rubber stoppers. Each hole has a glass tube and rubber hose attached. One hose is connected to a cold water source, the other to a drain outlet. With cold water circulating through the flask, which is inserted into a 1 L beaker containing the sample, the solvent is heated to a boil and allowed to reflux back into the sample for a set period of time. Multiple units should be set up in parallel with "T" connectors rather than in a series because increased temperature at the end of the series may affect the efficiency of the solvent reflux process.

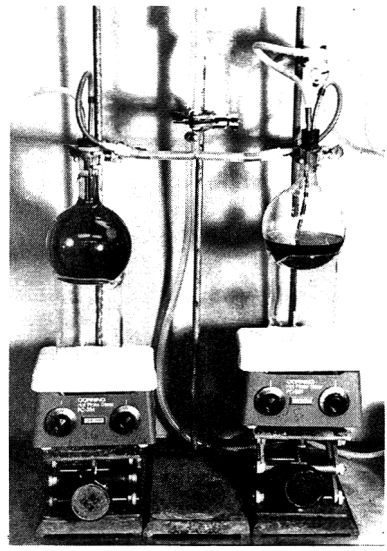


Figure 1. Solvent saver apparatus.

Solvent Saver Technique for Ground Turmeric and Oregano

(a) *Turmeric.*—Weigh sample into 1 L beaker. Add 400 mL isopropanol to sample beaker and boil gently on hot plate 30 min with solvent saver apparatus (Figure 1) inserted into beaker top. Pour sample into No. 230 sieve and wash with gentle stream of hot tap water. Proceed with extraction as in **44.123(b)**.

(b) *Oregano.*—Form filter paper cup (**44.022(k)**) with 400 mL beaker into 1 L beaker and weigh sample into cup. Add to 1 L beaker. Add 400 mL chloroform to cup in sample beaker and boil gently on hot plate-solvent saver apparatus in fume hood for 10 min. Transfer cup to buchner funnel and aspirate to slow drip. Turn off vacuum. Return cup to original beaker and

Received August 26, 1981. Accepted October 26, 1981.
This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.
The recommendation of the Associate Refereee was approved by the General Referee and Committee F and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) 65, 387.

0004-5756/82/6505-1089-04$01.00

cover sample with isopropanol. Boil on hot plate for 2 min without solvent saver apparatus. Remove paper cup and aspirate to near dryness. Turn off vacuum and proceed with extraction as in **44.123(b).**

Collaborative Study

Three collaborators each received 12 subsamples, 6 of ground oregano and 6 of ground turmeric. The collaborators examined 3 of the subsamples of each spice by the official pretreatment methods **44.122(a)** for turmeric and **44.122(b)** for oregano. The solvent saver technique was used to analyze the other 3 subsamples of each spice with the appropriate solvents. The same isolation method, **44.123(b),** was used for all subsamples. Each subsample was spiked with 20 elytral fragments ca 0.5 mm sq. and 20 rodent hair fragments ca 1–2 mm long. The collaborators reported their counts of the spike material and submitted the extraction plates to the Associate Referee for confirmation of the counts.

Results

This mini-collaborative study compared different defatting techniques for 2 previously studied spices, turmeric and oregano. Identical time frames and reagents were observed to provide a valid comparison. Statistical data in Tables 1–4 were calculated from the original studies (1, 2). Average recovery values obtained by the official method were similar in this study and in the original studies. Although variation was greater in this study, especially the reproducibility component, it was within acceptable limits.

For oregano, the solvent saver technique gave much lower recoveries of insect fragments than the official method (Table 1), and repeatability and reproducibility variations were much higher. There were no differences between the two methods in recoveries of rodent hairs (Table 2). For turmeric, recoveries of insect fragments and rodent hair fragments were almost identical by the solvent saver and official methods (Tables 3 and 4).

Discussion

The purpose of this study was to determine whether the solvent saver reflux technique could be used as an alternative to the official methods for defatting turmeric and oregano. When the solvent saver reflux technique was used for ground turmeric with isopropanol as the solvent, 66% of the solvent was saved, volatile vapors were confined to the solvent saver apparatus, and the economy and safety of the defatting were enhanced with no compromise in effectiveness.

The solvent saver reflux technique was also used for defatting ground oregano with chloroform as the solvent; however, recoveries of insect fragments were unsatisfactory compared with those of the official method.

These differences in insect fragment recoveries can be explained by the characteristics of the 2

Table 1. Collaborative results for the recovery [a] of insect fragments using official AOAC methods 44.122(b) and 44.123(b) for oregano with chloroform as the defatting agent

	Solvent saver (SS)			Official (O)		
Coll.	1	2	3	4	5	6
A	18(20)	8	15	20	17(18)	19
B	20	19(16)	18(17)	19	18	20
C	6(7)	9	5(4)	18	14(15)	20

	Associate Referee		Collaborators		Original study
Statistic	SS	O	SS	O	O
Av. recovery	12.9	18.5	12.0	18.3	18.7
% recovery	64.5	92.5	60.0	91.5	93.5
SD					
Repeatability	3.96	1.67	2.69	2.06	1.01
Reproducibility	6.50	1.67	6.70	2.06	1.06
CV, %					
Repeatability	30.70	9.00	22.42	11.26	5.40
Reproducibility	50.39	9.00	55.83	11.26	5.67

[a] Associate Referee counts are in parentheses.

Table 2. Collaborative results for the recovery of rodent hairs using official AOAC methods 44.122(b) and 44.123(b) for oregano with chloroform as the defatting agent

	Solvent saver (SS)			Official (O)		
Coll.	1	2	3	4	5	6
A	20	18	20	20	20	18
B	17	20	18	17	18	17
C	16	19	19	16	15	13

	Associate Referee		Collaborators		Original study
Statistic	SS	O	SS	O	O
Av. recovery	18.5	17.1	18.5	17.1	18.2
% recovery	92.5	85.5	92.5	85.5	91.0
SD					
Repeatability	1.49	1.16	1.49	1.16	1.03
Reproducibility	1.49	2.52	1.49	2.52	1.22
CV, %					
Repeatability	8.05	6.78	8.05	6.78	5.66
Reproducibility	8.05	14.74	8.05	14.74	6.70

solvents. The low boiling point of chloroform (61.15°C), its elevated vapor pressure (158.4 torr at 20°C), and its low solubility in water (0.056% at 20°C) enhance its defatting ability. Furthermore, in the reflux technique when the product is immersed in the solvent, the dissolved lipids tend to elevate the boiling point, further enhancing solvent action. By elevating the boiling point of the chloroform with solvated lipids, solvent cycling is increased. The effect of solvation on fats and waxes of the insect fragment cuticle, in turn, interferes with the filth extraction. Rodent hairs do not appear to be affected by either solvent.

Aside from having a higher boiling point (82.26°C), the other solvent used in this study, isopropanol, has a low defatting capability because it is highly soluble in water. Consequently, it is more suitable for defatting low fat spices without removing the fats and waxes from insect fragments which would ultimately interfere with the filth extraction process. Isopropanol, therefore, tends to be self-limiting in both defatting techniques.

Table 3. Collaborative results for the recovery [a] of insect fragments using official AOAC methods 44.122(a) and 44.123(b) for turmeric with isopropanol as the defatting agent

	Solvent saver (SS)			Official (O)		
Coll.	1	2	3	4	5	6
A	20	18	20	20	19	20
B	20	18	19	18	20	20
C	19	18	18	18	14	21(20)

	Associate Referee		Collaborators		Original study
Statistic	SS	O	SS	O	O
Av. recovery	18.9	18.8	18.9	18.9	19.2
% recovery	94.5	94.0	94.5	94.5	96.0
SD					
Repeatability	0.94	0.94	1.92	2.16	0.93
Reproducibility	0.94	2.01	0.94	2.16	1.00
CV, %					
Repeatability	4.97	10.21	4.97	11.43	4.84
Reproducibility	4.97	10.69	4.97	11.43	5.21

[a] Associate Referee counts are in parentheses.

Table 4. Collaborative results for the recovery of rodent hairs using the official AOAC methods 44.122(a) and 44.123(b) for turmeric with isopropanol as the defatting agent

	Solvent saver (SS)			Official (O)		
Coll.	1	2	3	4	5	6
A	18	20	20	20	17	19
B	20	18	19	19	19	20
C	12	20	13	14	15	16

	Associate Referee		Collaborators		Original study
Statistic	SS	O	SS	O	O
Av. recovery	17.8	17.7	17.8	17.7	17.5
% recovery	89.0	88.5	89.0	88.5	87.5
SD					
Repeatability	2.67	1.11	2.67	1.11	0.97
Reproducibility	3.25	2.50	3.25	2.50	0.97
CV, %					
Repeatability	15.00	6.27	15.00	6.27	5.54
Reproducibility	18.26	14.12	18.26	14.12	5.54

Recommendations

When used with isopropanol as a defatting agent, the solvent saver technique can be an alternative to the official method for turmeric, **44.122(a)** and **44.123(b)**, with little or no loss in insect fragment and rodent hair fragment recoveries. It is recommended that the solvent saver technique be inserted in the AOAC *Official Methods of Analysis*. Because of changes in solvent characteristics and low insect fragment recoveries, the solvent saver technique was unsuccessful with chloroform and therefore is not recommended for oregano. Further investigations of the solvent saver technique should be made during development of methods which require a defatting pretreatment.

Acknowledgments

The author thanks the following collaborators, all of the Food and Drug Administration: R. B. Bradicich, Dallas, TX; G. Dzidowski, Detroit, MI; L. Glaze, Division of Microbiology, Washington, DC; J. Quaife, Minneapolis, MN. The author is also grateful to the following, all of the Food and Drug Administration, Bureau of Foods, Washington, DC: C. R. Warner and F. McClure for technical and statistical assistance, J. L. Boese and J. S. Gecan for reviewing the manuscript, and S. M. Cichowicz for photography.

References

(1) Glaze, L. E. (1975) *J. Assoc. Off. Anal. Chem.* **65**, 447–450

(2) Glaze, L. E. (1975) *J. Assoc. Off. Anal. Chem.* **65**, 451–452

Howard Mold Count of Fruit Nectars, Purees, and Pastes: Collaborative Study

STANLEY M. CICHOWICZ and RUTH BANDLER
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Collaborators: R. Bandler; G. Dzidowski; D. Floyd; R. Galacci; J. Kaminski; F. McNerney

The various mold count methods for nectars, purees, and pastes make no adjustment for concentration of the pulped liquid. Thus, mold counts of different forms of the same product (e.g., guava nectar, puree, or paste) or of the same product from different manufacturers cannot be compared. A method was developed in which the product is centrifuged under standardized conditions, then diluted to a standard pellet-to-final-volume ratio. Because variations in the concentration of fruit or vegetable tissue are corrected, comparisons of mold counts are possible. The method was adopted official first action.

The primary difference among fruit nectars, purees, and pastes is concentration. Traditionally, mold counts of comminuted fruit products **(44.096)** made no allowance for variations in concentration. Samples of peach nectar prepared for mold counting by the official method were extremely thin with virtually no fruit tissue, which is the source of mold to be counted; samples of infant peach puree, on the other hand, had a high fruit tissue concentration. Furthermore, addition of sugar made concentration adjustment of soluble solids impossible.

In the proposed method, the plant tissue concentration was standardized by diluting a centrifuged pellet by volume with stabilizer solution. Comparison of mold counts of different products of the same fruit (e.g., nectar, puree, or paste) or of the same product from different manufacturers was thus possible.

Howard Mold Count of Fruit Nectars, Purees, and Pastes

Official First Action

44.C08 *Sample Preparation*

(a) *Fruit nectars.*—Measure 40 mL well mixed sample into 40 mL graduated, thick-wall centrf. tube (Corning, Pyrex No. 8340, or equiv.) and proceed as in **44.C09.**

(b) *Fruit purees with no added starch.*—Dil. sample 1 + 1 with H_2O, measure 40 mL well mixed sample into 40 mL graduated, thick-wall centrf. tube, and proceed as in **44.C09.**

(c) *Fruit purees with added starch.*—Weigh 50 g fruit puree into beaker and add 50 mL HCl soln (5 + 45). Mix well and heat on steam bath 15 min. Measure 40 mL well mixed, hydrolyzed sample into 40 mL graduated, thick-wall centrf. tube and proceed as in **44.C09.**

(d) *Fruit pastes.*—Disperse 1 part paste in 3 parts H_2O. If necessary, warm gently to break gel. Measure 40 mL well mixed sample into 40 mL graduated, thick-wall centrf. tube and proceed as in **44.C09.**

44.C09 *Centrifugation and Concentration Adjustment*

Centrf. 10 min at 2200 rpm as in **44.082.** Gradually let centrf. come to complete stop. Remove tubes and immediately decant supernate without disturbing sediment. Gently tap centrf. tube to level top of sediment. Dil. sediment with stabilizer soln, **44.003(gg),** as follows: (*1*) peach, apricot, mango, and papaya: 1 + 1; (*2*) pear and guava: 1 + 3; (*3*) strawberries, blackberries, raspberries, and blueberries: 1 + 6.

Proceed with Howard mold count as in **44.096,** beginning "Clean Howard cell . . ."

Collaborative Study

Six collaborators each examined 6 samples of peach nectar. All aliquots were taken from a homogeneous mixture spiked with *Rhizopus* mold (chosen for its coarse, easily recognizable hyphae). The mold spike was grown in a 250 mL screw-cap flask containing 125 mL sterile peach nectar. The culture medium was inoculated with *Rhizopus* mold and incubated at room temperature on a shaker for 3 days.

After incubation, 15 mL formalin was added to the culture flask. The mixture was transferred to a Sorvall Omni-Mixer fitted with a 600 mL cup

Received June 30, 1981. Accepted October 26, 1981.
This report of the Associate Referee, S. M. Cichowicz, was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.
The recommendation of the Associate Referee was approved by the General Referee and Committee F and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) 65, 386.

Table 1. Mold counts of peach nectar by collaborators

	Sample					
Coll.	1	2	3	4	5	6
A	40	50	41	44	41	42
B	53	50	53	50	50	50
C	45	33	48	37	33	45
D	45	47	61	47	51	34
E	33	26	46	38	41	38
F	46	48	46	41	51	38
Av.		44.222				
SD						
Repeatability		5.464				
Reproducibility		6.790				
CV, %						
Repeatability		12.36				
Reproducibility		15.35				

and blended at ca 13 000 rpm for 3 min. The spiking mixture was added in 20 mL portions to a 3 L beaker containing 2.5 L peach nectar and mixed well. The mold count was checked until the desired mold count range was reached.

Results and Discussion

Few problems were encountered in the sample preparation and counting procedure. The collaborators' counts are presented in Table 1. Traditionally, the Associate Referee counts the collaborators' preparations; however, the Referee's counts were unnecessary in this study. The low coefficients of variation (repeatability 12.36% and reproducibility 15.35%) presented in Table 1 support this decision. In the past, analyst counts yielded coefficients of variation of 50% or more in similar microscopic procedures, probably because collaborative studies were assigned without regard to the analysts' training or experience in mold counting.

This collaborative study tested the centrifugation technique in the sample preparation. The study was not intended to test the analysts' abilities to count mold; however, the choice of trained analysts and the use of coarse mold for spiking the samples undoubtedly added to the success of this study.

Recommendation

It is recommended that the proposed method be adopted official first action for fruit nectars, purees, and pastes.

Acknowledgments

The help of the following analysts, all of the Food and Drug Administration, is gratefully acknowledged: Ruth Bandler, Division of Microbiology, Washington, DC; Gary Dzidowski, Detroit, MI; Deborah Floyd, Dallas, TX; Raymond Galacci, Baltimore, MD; Jane Kaminski, San Francisco, CA; Francis McNerney, Buffalo, NY.

Determination of *Geotrichum* Mold in Comminuted Fruits and Vegetables: Collaborative Study

STANLEY M. CICHOWICZ and RUTH BANDLER
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Collaborators: R. Bandler; G. Dzidowski; D. Floyd; J. Kaminski; F. McNerney; R. Trauba

The official AOAC method for determination of *Geotrichum* mold in canned fruits and vegetables (44.079) requires a series of 3 sieves, Nos. 8, 16, and 230, to separate the packing liquid from the product and the mold from the packing liquid. Although this method has been successful for whole or coarsely chopped products (e.g., green beans, potatoes, carrots, and beets), finely divided products such as fruit purees and tomato products tend to clog the sieves. A method was developed in which the product is centrifuged, diluted by volume, stained with crystal violet, and counted with the sieving steps eliminated. The proposed method was adopted official first action.

The official methods for determination of *Geotrichum* mold in fruit and vegetable products, **44.079** and **44.106**, use a series of sieves, Nos. 8, 16, and 230, for the separation and concentration of mold fragments. The method is intended for coarse products that consist of large pieces of intact fruits or vegetables packed in liquid which contains few suspended pieces of finely divided plant tissue. When used to prepare a comminuted fruit such as infant peach puree, the finely divided product clogged No. 230 sieve. The small pieces of fruit tissue could not be separated from the liquid by sieving.

The proposed method eliminates all sieving. The sample is stained, diluted if it is a heavy paste or puree, and centrifuged. The supernatant liquid is decanted, and the pellet of plant tissue is diluted by volume with stabilizer solution. The sample is counted as in **44.079(d)**. The centrifugation step allows standardization of tissue concentration before examination.

Collaborative Study

Six collaborators each examined 6 samples of peach nectar. All aliquots were taken from a homogeneous mixture spiked with naturally occurring machinery mold.

This report of the Associate Referee, S. M. Cichowicz, was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee F and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) **65**, 386.

Received June 30, 1981. Accepted October 26, 1981.

Geotrichum Mold in Comminuted Fruits and Vegetables

Official First Action

44.C06 ***Sample Preparation***

(a) *Fruit nectars.*—Add 40 mL nectar and 10 drops of crystal violet stain, **44.003(p)**, to 40 mL centrf. tube (Corning, Pyrex No. 8340, or equiv.). Mix well and proceed as in **44.C07.**

(b) *Purees with no added starch.*—Add 20 mL puree and 10 drops of crystal violet stain, **44.003(p)**, to 40 mL centrf. tube. Mix well. Bring vol. to 40 mL with H_2O and mix well. Proceed as in **44.C07.**

(c) *Purees with starch added.*—Add 50 mL HCl soln (5 + 45) to 50 g fruit puree. Mix well and heat with mag. stirring until starch clears. Neutze soln with 50% KOH or 50% NaOH to pH 7.0 ± 1.0. Transfer 40 mL soln to 40 mL centrf. tube and add 20 drops of crystal violet stain, **44.003(p).** Mix well and proceed as in **44.C07.**

(d) *Pastes.*—Disperse 1 part paste in 3 parts H_2O. If necessary, warm gently to break gel. Transfer 40 mL soln to 40 mL centrf. tube and add 10 drops of crystal violet stain, **44.003(p).** Mix well and proceed as in **44.C07.**

44.C07 ***Centrifugation***

Centrf. 10 min as in **44.082.** Immediately after centrf. comes to rest, decant aq. layer and read vol. of sediment. Dil. sediment 1 + 3 (v/v) with stabilizer soln, **44.003(gg).**

Pipet and count as in **44.079(d).** Express results in mycelial fragments per 100 mL prepn.

Results and Discussion

The official AOAC methods, **44.079** and **44.106**, are not applicable to comminuted fruits and vegetables because the finely divided products clog the No. 230 sieve. Samples prepared by the proposed method contain a great amount of masking plant tissue, which makes the mold hard to find and increases the counting time. However, the centrifugation allows tissue concentration of the different forms of each product

0004-5756/82/6505-1095–02$01.00

Table 1. Counts of *Geotrichum* mold in peach nectar by collaborators and Associate Referee

Coll.	Sample 1	2	3	4	5	6
A	76(87)[a]	72(86)	106(99)	121(94)	97(125)	96(96)
B	42(111)	43(121)	44(80)	45(97)	42(86)	44(102)
C	105(100)	113(115)	109(108)	114(76)	98(122)	82(112)
D	78(97)	67(90)	142(96)	107(97)	69(110)	83(107)
E	20(105)	19(110)	24(121)	19(122)	18(94)	23(80)
F	69(104)	79(100)	125(110)	115(83)	112(109)	111(82)
Av.			75.806(100.917)			
SD						
Repeatability			17.417(14.035)			
Reproducibility			38.492(14.035)			
CV, %						
Repeatability			22.98 (13.91)			
Reproducibility			50.78 (13.91)			

[a] Associate Referee counts of the same samples are in parentheses.

(i.e., nectars, purees, and pastes) to be standardized before examination.

The collaborators' counts and the Associate Referee's counts of the collaborators' preparations are presented in Table 1. On average, the collaborators' counts were lower than those of the Associate Referee (75.806 vs 100.917), probably because some analysts lacked training and practice and because of relatively large amounts of masking plant tissue. The collaborators' counts also varied more than those of the Associate Referee, as shown by the repeatability and reproducibility values. The low coefficient of variation (13.91) showed that good precision was attained by analysts who received adequate training and practice in performing the method.

Recommendation

It is recommended that the proposed method be adopted official first action for comminuted fruits and vegetables.

Acknowledgments

The help of the following analysts, all of the Food and Drug Administration, is gratefully acknowledged: Ruth Bandler, Division of Microbiology, Washington, DC; Gary Dzidowski, Detroit, MI; Deborah Floyd, Dallas, TX; Jane Kaminski, San Francisco, CA; Francis McNerney, Buffalo, NY; Richard Trauba, Minneapolis, MN.

PESTICIDE RESIDUES

High Performance Liquid Chromatographic Determination of Difenzoquat Residues in Water

IJAZ AHMAD
Alberta Environmental Centre, Vegreville, Alberta, Canada T0B 4L0

A simple analytical method is described for the quantitative determination of difenzoquat (1,2-dimethyl-3,5-diphenyl-1*H*-pyrazolium methyl sulfate) residues in water. The method involves high performance liquid chromatography with ultraviolet detection at 255 nm. The procedure is used to determine 2 ppb to 1 ppm levels of difenzoquat in pure and tap water. Samples which contain ≥50 ppb difenzoquat are injected directly into the liquid chromatograph without prior separation, concentration, or purification. Samples containing ≤50 ppb difenzoquat are first enriched on C_{18} Sep-Pak reverse phase cartridges and then injected into the liquid chromatograph. Average recoveries of difenzoquat were greater than 92% with a coefficient of variation less than 4.5%. The method can be used for water samples with difenzoquat concentrations as low as 2 ppb.

Difenzoquat (1,2-dimethyl-3,5-diphenyl-1*H*-pyrazolium methyl sulfate) is an herbicide used throughout the world to control wild oats. It is a quaternary ammonium salt which is highly soluble in water and partitions strongly in the favor of water. It has a negligible vapor pressure and is quite stable to hydrolytic or biological degradation (1). Difenzoquat is the active ingredient in Avenge® and Finaven® (registered trademarks of American Cyanamid Co.). A 26.9% solution and a 51.7% soluble powder are registered in Canada under the Pest Control Product Act.

Analysis of difenzoquat in commercial formulations has been described in the literature (2, 3). Steller (4) has recommended a gas-liquid chromatographic method validated at levels as low as 50 ppb difenzoquat in soil, plant, and animal tissues. This paper describes a procedure for the quantitative determination of difenzoquat residues in water. The method is simple, quick, and reliable, and permits rapid monitoring of difenzoquat in water.

Received December 2, 1981. Accepted February 22, 1982.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Perkin-Elmer Series II with Rheodyne sample injector Model 7120 (with 175 μL loop) or Model 7150 equipped with Perkin-Elmer variable wavelength ultraviolet (UV) detector, Model LC-55, and Hewlett-Packard integrator Model 3380A. Typical chromatographic conditions: temperature, ambient; flow rate, 2 mL/min; wavelength, 255 nm; chart speed, 0.5 cm/min; injection volume, 175 μL with 175 μL loop.

(b) *Chromatographic column.*—Whatman Partisil PXS 10/24 ODS, stainless steel, 25 cm long × 4.6 mm id (Whatman Inc., Clifton, NJ 07014).

(c) *Guard column.*—Stainless steel, 7.0 cm long × 4.6 mm id, laboratory-packed with Co:Pell ODS (Whatman Inc.).

(d) *Reagents.*—Difenzoquat (analytical grade 98%+ purity) was supplied by the Laboratory Services Division, Food Production and Marketing Branch, Agriculture Canada, Ottawa, Ontario. Analytical grade potassium dihydrogen orthophosphate (BDH Chemical), reagent grade 85% phosphoric acid (American Scientific and Chemical), and pesticide grade acetonitrile (Fisher Scientific Co.) were obtained from local suppliers. Pure water, used throughout this work, was prepared by passing distilled water through a Milli-Q™ water purification system (Millipore Co., Bedford, MA 01730).

Tap water for spiking purposes was collected in April 1981 from a water tap located in the Alberta Environmental Centre, Vegreville, Alberta.

(e) *Mobile phase.*—See figure captions.

(f) *Eluant solution.*—Dissolve 6.8045 g potassium dihydrogen orthophosphate in 500 mL 50% acetonitrile–water, and adjust pH of resulting solution to 2.80 with phosphoric acid.

(g) *Stock solution.*—1 μg difenzoquat/μL. Accurately weigh 100 mg difenzoquat, dissolve in water in 100 mL volumetric flask, and dilute to volume with water.

0004-5756/82/6505-1097-05$01.00

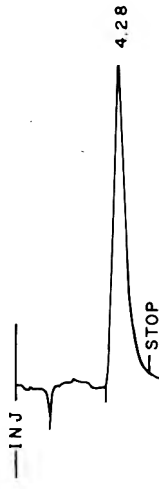


Figure 1. Chromatogram of 1.00 ppm difenzoquat in pure water.

Chromatographic conditions: mobile phase, 6.8045 g potassium dihydrogen orthophosphate in 500 mL 60% acetonitrile–water, pH adjusted to 4.01 with phosphoric acid; injection volume, 175 μL; flow rate, 2 mL/min; UV wavelength, 255 nm; chart speed, 0.5 cm/min; attenuation, 8; temperature, ambient.

Preparation of Spiked Samples

Transfer 0.0, 1.0, 2.5, 5.0, 10.0, 12.5, 25.0. 50.0, 100.0, 250.0, and 500.0 μL portions of stock solution with the appropriate syringe into separate clean 500 mL volumetric flasks. Dilute each to 500 mL with pure or tap water. Concentrations of difenzoquat in above samples are 0.0, 2.0, 5.0, 10.0, 20.0, 25.0, 50.0 ppb, and 0.10, 0.20, 0.50, and 1.00 ppm, respectively.

Determination

Water samples spiked with 0.05–1.00 ppm difenzoquat.—Filter sample through 0.45 μm MF-Millipore filter (HA WP 01300) with sample preparation filtration kit (Millipore Co.), and inject 175 μL filtrate into liquid chromatograph. Make 3 replicate injections and calculate amount of difenzoquat by external standard method.

Water samples spiked with 2–50 ppb difenzoquat.—Dissolve 1.70 g potassium dihydrogen orthophosphate in 500 mL spiked sample and filter resulting solution through 0.45 μm MF-Millipore filter (HA WP 04700) with all-glass filtration apparatus (Millipore Co.).

Pass 5 mL eluant solution through C_{18} Sep-Pak cartridge (Waters Associates, Inc., Milford, MA 01757) at ca 2 mL/min.

Quantitatively pass 100 mL (or as required) of above filtrate through pre-washed C_{18} Sep-Pak cartridge at 2.5 mL/min.

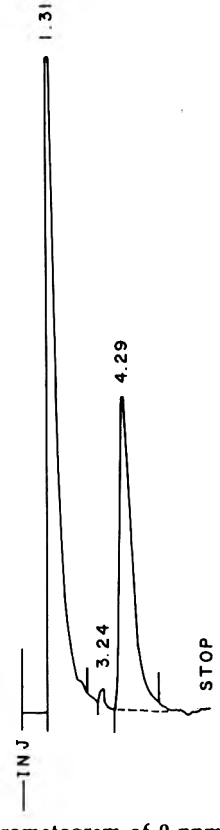


Figure 2. Chromatogram of 0 ppm (---) and 1.00 ppm difenzoquat in Vegreville tap water samples. Chromatographic conditions as given in Figure 1.

Drain and discard water from cartridge, and elute difenzoquat with eluant solution at ca 1.0 mL/min. Collect first 5 mL eluate in 5 mL volumetric flask.

Make 3 replicate 175 μL injections of concentrated sample into liquid chromatograph and calculate amount of difenzoquat by external standard method.

Results and Discussion

Water samples (pure or tap water) spiked at 0.05–1.00 ppm levels were injected directly into the liquid chromatograph by using a sample injector equipped with the 175 μL loop. No major sample preparation was required except filtration of the small volume of water sample through a 0.45 μm filter to remove particulate material which might plug the column, hydraulic lines, or in-line filters. In the present work, sample filtration and injection into the liquid chromatograph was accomplished simultaneously by

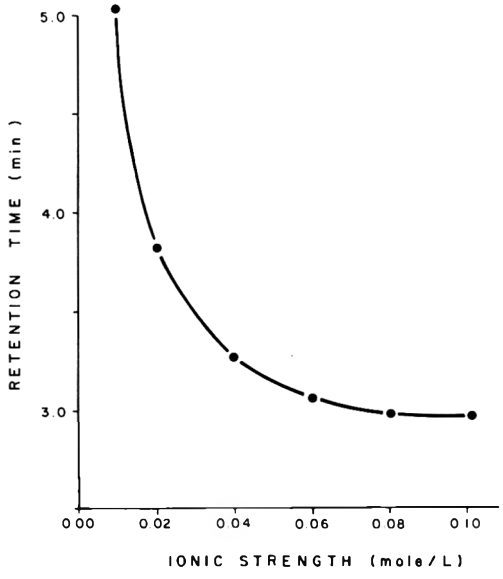


Figure 3. Effect of variation of eluant ionic strength on retention time of difenzoquat.

Chromatographic conditions: mobile phases: mix amounts of solution I (6.8050 g potassium dihydrogen orthophosphate in 500 mL 50% acetonitrile–water adjusted to pH 3.00 with phosphoric acid) and solution II (50% acetonitrile–water adjusted to pH 3.00 with phosphoric acid) to achieve desired concentration of potassium dihydrogen orthophosphate in mobile phase; injection volume, 2 μL (100 ng of difenzoquat); flow rate, 2 mL/min, UV wavelength, 255 nm; chart speed, 0.5 cm/min; temperature, ambient.

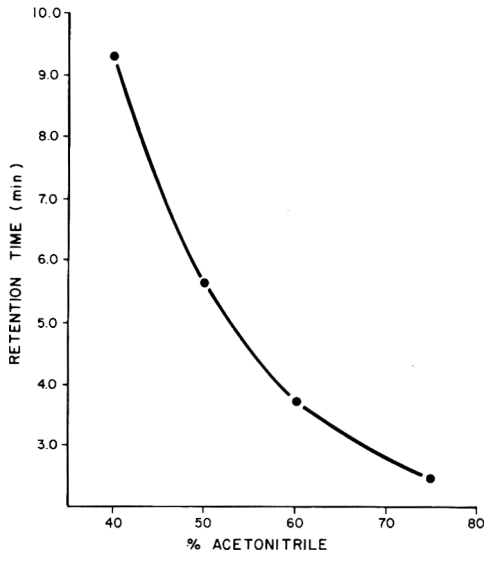


Figure 4. Effect of variation of eluant acetonitrile concentration on retention time of difenzoquat.

Chromatographic conditions: mobile phase, 0.6805 g potassium dihydrogen orthophosphate in 500 mL 40–80% acetonitrile–water adjusted to pH 2.80 with phosphoric acid, other conditions as given in Figure 3.

using the sample preparation kit. The Luer end of the syringe barrel was fitted with an adaptor for injection into the sample injector for LC determination. Figures 1 and 2 are chromatograms of 175 μL (175 ng) of 1 ppm difenzoquat in pure and tap water samples, respectively. The peaks at 1.31 and 3.24 min in Figure 2 were due to some unknown impurities present in the tap water sample.

Within-run precision was calculated on the basis of 5 replicate samples of pure water spiked at 0.25 ppm difenzoquat. The average recovery of difenzoquat from these samples was 98%; the coefficient of variation was 2.0%.

The linearity of UV detector response for difenzoquat was checked. Results were plotted as peak area vs concentration of difenzoquat in the pure and tap water samples. The calibration graphs showed no significant difference. The curves were linear over the concentration range examined (pure water, $r = 0.999$; tap water, $r = 0.998$), and gave a lower limit of detection of 0.05 ppm or 8.75 ng/175 μL of the injected material (signal-to-noise ratio = 2).

The effects of eluant ionic strength, acetonitrile concentration, and pH on the retention of difenzoquat on the C_{18} reverse phase analytical column were studied. The results are shown in Figures 3–5. Decreasing the ionic strength, acetonitrile concentration, and/or increasing the pH of the mobile phase increased the retention of difenzoquat; the reverse was also true. The study was helpful in selecting the composition of the mobile phase to achieve good separation of difenzoquat in the presence of impurities.

Water samples spiked at 2–50 ppb levels of difenzoquat were studied by extracting the difenzoquat from these samples and then analyzing the extracts by high performance liquid chromatography. The method for extracting difenzoquat from these samples was based on the affinity of ion pairs for reverse phase chromatographic support. The basic theory of the ion-pair technique and its practical aspects have been discussed (5, 6). Potassium dihydrogen orthophosphate was used as an ion-pair reagent, and was added to each water sample. A Mini-Pump® (Milton Roy Co., St. Petersburg, FL) was used to pass the appropriate amount of water sample

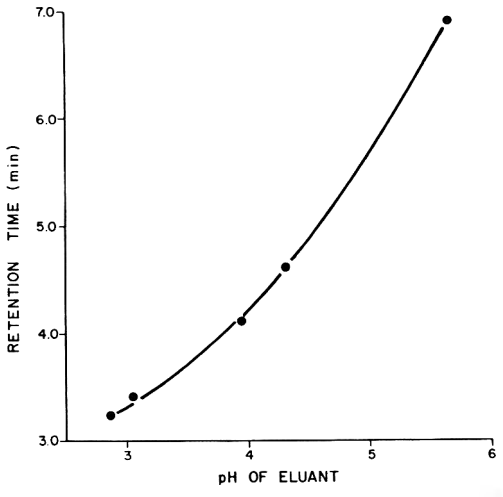


Figure 5. Effect of variation of eluant pH on retention time of difenzoquat.

Chromatographic conditions: mobile phase, 0.6805 g potassium dihydrogen orthophosphate in 500 mL 60% acetonitrile–water adjusted to pH 2.80–5.80 with phosphoric acid; other conditions as given in Figure 3.

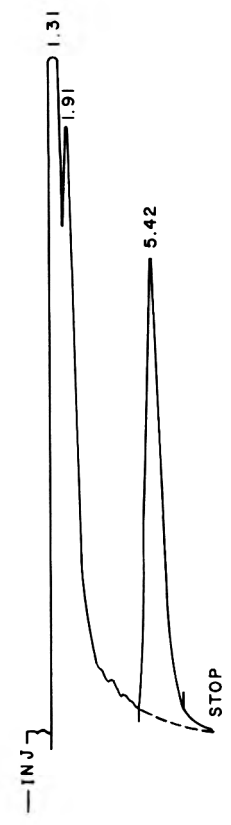


Figure 6. Chromatogram of 0 ppb (---) and 50 ppb difenzoquat in tap water.

Chromatographic conditions: mobile phase, 0.02M potassium dihydrogen orthophosphate–acetonitrile (50 + 50) adjusted to pH 3.02 with phosphoric acid; injection volume, 175 μL; flow rate, 2 mL/min; UV wavelength, 255 nm; chart speed, 0.5 cm/min; attenuation, 4; temperature, ambient.

through the C_{18} cartridge. Any other appropriate device for passing a larger volume of sample could also be used for this purpose. After sufficient concentration, the difenzoquat was desorbed from the cartridge with the eluant solution and then analyzed by high performance liquid chromatography. The treatment (washing) of the cartridge with the eluant solution was necessary before the concentration step. No difenzoquat was adsorbed on the cartridge when any of the above spiked samples (without the addition of ion pair reagent, KH_2PO_4) were passed through the untreated (new) cartridge. Figure 6 is a chromatogram of 0 and 50 ppb difenzoquat in tap water samples. The peaks at 1.31 and 1.91 min were due to some unknown impurities present in the tap water. An attempt to eliminate such impurities by washing the cartridge with 10–50 mL ether–hexane (20 + 80) before desorption was not successful. Recoveries of difenzoquat from the pure and tap water samples (Table 1) were greater than 92% with a coefficient of variation of less than 4.3%.

To determine within-run precision, 5 pure water samples were spiked at 20 ppb difenzoquat, and carried through the procedure. The average recovery of difenzoquat from these samples was 95%, and the coefficient of variation was 4.1%.

The calibration curve between peak area and difenzoquat concentration (Table 1) was linear over the concentration range examined and gave a correlation coefficient of 0.999; this linearity showed that the method was well suited for the determination of difenzoquat in water. The recovery values listed in Table 1 reveal the high efficiency of the method.

The method was also tested with Vermilion River water which was fortified with difenzoquat. The results were similar to those for Vegreville tap water samples.

This method presents a rapid, accurate procedure for determining difenzoquat in water and has a potential for application to analysis of other pesticide residues.

Table 1. Recovery of difenzoquat from fortified pure and tap water samples[a]

Sample	Fortification level, ppb	Vol. sample concd, mL	Difenzoquat present, μg	Pure water		Tap water	
				Rec, μg	Rec., %	Rec, μg	Rec., %
5	50	100	5.0	4.83	98.6	4.63	92.6
6	25	100	2.5	2.22	88.8	2.48	99.2
7	20	100	2.0	1.88	94.0	1.89	94.5
8	10	100	1.0	0.89	89.0	0.90	90.0
9	5	100	0.5	0.49	98.0	0.47	94.0
10	2	200	0.4	0.36	90.0	0.36	90.0
Av.					92.7		93.4
SD (n = 6)					4.0		3.4
CV, %					4.3		3.6

[a] Each value is an average of 3 replicate determinations. Chromatographic conditions are given in Figure 6.

Acknowledgment

The author thanks the Laboratory Services Division, Food Production and Marketing Branch, Agriculture Canada, Ottawa, Ontario, for providing an analytical standard of difenzoquat.

References

(1) Kapoor, I. P., & Boyd, J. E. (1977) *West Soc. Weed Sci. Proc.* **30**, 96–97

(2) Barry, C., & Pike, R. K. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 647–649

(3) *Technical Report on Avenge®*, American Cyanamid Co., Princeton, NJ, pp. 8–9

(4) Steller, W. A. (1980) *Analytical Methods for Pesticides and Plant Growth Regulators*, Vol. XI, Academic Press, Inc., New York, NY, pp. 291–305

(5) Schill, G. (1976) *Assay of Drugs and Other Trace Compounds in Biological Fluid*, E. Reid (Ed.), North Holland Publishing Co., New York, NY

(6) Gloor, R., & Johnston, E. L. (1977) *J. Chromatogr. Sci.* **15**, 413–423

Modified Gas-Liquid Chromatographic Method for Determination of Compound 1080 (Sodium Fluoroacetate)

IWAO OKUNO, DENNIS L. MEEKER, and ROBERT R. FELTON
U.S. Fish and Wildlife Service, Denver Wildlife Research Center, Denver Federal Center, Denver, CO 80225

A method capable of determining 0.1 ppm 1080 (sodium fluoroacetate) in 1 g animal tissue was developed. It involves extraction of 1080 from the sample with acetone-water, and then evaporation of the acetone followed by extraction of 1080 as fluoroacetic acid from water with ethyl acetate. Ethyl acetate is removed by volatilization from fluoroacetic acid which is retained as the triethanolammonium fluoroacetate salt. Fluoroacetic acid is subsequently derivatized with α-bromo-2,3,4,5,6-pentafluorotoluene and quantitated by gas-liquid chromatography with an electron capture detector. The method is rapid and requires no special apparatus or equipment and no more than 12 mL of any one solvent. Recoveries of 1080 from tissue samples fortified with 0.1–100 ppm averaged about 85%.

Several gas-liquid chromatographic (GLC) methods (1–6) and, more recently, high performance liquid chromatographic (HPLC) methods (7, 8) have been reported for the determination of 1080 (sodium fluoroacetate). These methods require some type of derivatization of sodium fluoroacetate, either because of poor chromatographic properties of the conjugate fluoroacetic acid or because of limited detection of the acid by the detector systems used. Most GLC methods involve conversion of the acid to an alkyl ester for chromatographic separation and measurement with a flame ionization detector (1–4) or by mass spectrometry (5). Flame ionization detection procedures lack sensitivity, and mass spectrometry is not readily available for routine use in most laboratories. In HPLC methods, 1080 is derivatized with *o-p*-nitrobenzyl-*N-N'*-diisopropylisourea and measured with an ultraviolet (UV) detector (7) or measured by fluorescence as the derivative of 4-bromo-methyl-7-methoxycoumarin (8). Based on the minimum amount of derivative detected in a standard solution, a sensitivity greater than 0.1 ppm was estimated for the UV method but recovery data were not presented for levels below 1 ppm. In the fluorescence procedure, a detection limit of 0.2 ng sodium fluoroacetate as the derivative was reported but the lowest 1080 spike level tested with samples was 100 mg/kg.

Previously, we reported a GLC method for the determination of 1080 in which it is derivatized with α-bromo-2,3,4,5,6-pentafluorotoluene (pentafluorobenzyl bromide, PFBB) and measured with an electron capture detector (6). Although the sensitivity of the method was about 0.2 ppm, recoveries were only in the 20–30% range. The low recoveries resulted from losses incurred in the cleanup procedure which consisted of a sequence of silica gel adsorption and distillation steps to separate fluoroacetic acid from co-extracted water. We have since modified the method by eliminating the 2 steps and as a result have substantially improved recoveries to about 90%. In addition to the improved recoveries, the method is considerably simpler and requires less time.

METHOD

Reagents and Apparatus

(a) *Solvents.*—Analytical reagent acetone (Sargent-Welch, Skokie, IL 60077); distilled-in-glass hexane and 2,2,4-trimethylpentane (Burdick & Jackson Laboratories Inc., Muskegon, MI 49442); nanograde ethyl acetate (Mallinckrodt Inc., Paris, KY 40361).

(b) *Solutions.*—Sodium fluoroacetate (Sigma Chemical Co., St. Louis, MO 63178), 1000 μg/mL standard solution in acetone-water (9 + 1); α-bromo-2,3,4,5,6-pentafluorotoluene (Aldrich Chemical Co., Milwaukee, WI 53233), 10% solution in 2,2,4-trimethylpentane; Baker Analyzed reagent triethanolamine (J. T. Baker Chemical Co., Phillipsburg, NJ 08865), 10% solution in acetone.

(c) *Gas chromatograph.*—Tracor MT-220 equipped with ^{63}Ni detectors and EC linearizers. Operating conditions: inlet temperature 150°C, detector 350°C, column temperatures and carrier gas (10% methane in argon) flow rates specified with chromatographic columns.

(d) *Gas chromatographic columns and conditions.*—Three 1.8 m × 2 mm id glass columns. Column packing, temperature, and carrier gas

Received October 27, 1981. Accepted February 9, 1982.

Use of trade names does not imply endorsement of commercial products by the United States Government.

0004-5756/82/6505-1102-04$01.00

flow rate: (*1*) 5% QF-1 on 100–120 mesh Chromosorb W DMCS, 90°C, 35 mL/min; (*2*) 1:1 mixture of 5% QF-1 and 3% DC-200/12,500 on 100–120 mesh Chromosorb W DMCS, 90°C, 25 mL/min; (*3*) 0.5% OV-210 + 0.65% OV-17 on 100–120 mesh Ultrabond 20M (Ultra Scientific Corp., 1 Main St, Hope, RI 02831), 100°C, 20 mL/min. Columns should be freshly packed and used exclusively for 1080 analyses. Column life is indefinite under normal use but reduced column performance may result if used for other analyses.

Procedure

Sample extraction and cleanup.—Weigh 1 g homogeneously ground tissue sample into 20 × 120 mm screw-cap culture tube, add 4 mL acetone–water (8 + 2), cap (Teflon-lined), mix in ultrasonic bath ≥15 min, centrifuge, and decant supernate into 15 × 110 mm screw-cap tube. Repeat extraction of sample twice with same volume of solvent. Place tube in hot water bath (≤65°C to prevent bumping) and reduce volume to ca 2 mL with gentle stream of nitrogen. Partition aqueous solution with 3 mL hexane, centrifuge, and draw off and discard hexane with hypodermic syringe or aspirator. Repeat twice with same amount of hexane. Add 0.2 mL 1N HCl to aqueous solution. If sample is suspected of being highly alkaline, measure pH of aqueous solution and adjust to pH ≤2. Extract (shake vigorously 2–3 min) aqueous layer with 3 mL ethyl acetate, centrifuge, transfer ethyl acetate with clean hypodermic syringe to clean 15 × 110 mm screw-cap tube. Repeat extraction 3 times with same amount of ethyl acetate. When transferring ethyl acetate, let a small amount remain so that absolutely none of the lower aqueous layer is included in the transfer. Add 100 μL triethanolamine–acetone solution to ethyl acetate, mix, place in hot water bath (ca 70°C), and evaporate to dryness with a stream of nitrogen. Add 5 mL acetone to tube containing sample, cap (Teflon-lined, gas-tight), and mix in ultrasonic bath ≥5 min. If sample solution contains ≤100 μg 1080, proceed to derivatization step. If sample solution is suspected of containing a larger quantity of 1080, dilute aliquot containing ≥100 μg to 5 mL; then proceed to derivatization step.

Derivatization and gas chromatographic analysis.—Add 0.1 g K_2CO_3 (reagent, anhydrous, granular), 0.5 g Na_2SO_4 (reagent, anhydrous, granular), and 100 μL 10% PFBB solution. For diluted sample solution, use proportionately less (but ≥20 μL) 10% PFBB. Cap tightly, vortex mix, and heat at 55°C in tube heater (or equivalent) for 30 min.

Prepare 0.2–2 μg/mL working standard solutions by derivatizing 1–10 μL aliquots of 1000 μg sodium fluoroacetate standard/mL. For <0.2 μg/mL working standards, derivatize appropriate aliquots of 100 μg/mL standard (1000 μg/mL standard diluted 1:10 with acetone). Place aliquot in 15 × 110 mm screw-cap tube containing 5 mL acetone, add K_2CO_3 and Na_2SO_4 as for sample but only 20 μL PFBB solution, cap, vortex mix, and heat with samples at 55°C for 30 min. Do not derivatize more than 50 μL of 1000 μg/mL sodium fluoroacetate standard, to avoid introducing an excessive amount of water which interferes in the derivatization reaction.

Analyze derivatized sample solution on 2 gas chromatographic columns. For routine analysis, use mixed QF-1/DC-200 and Ultrabond 20M columns; QF-1 column can be used as an alternative column. Inject 1–2 μL sample solution, and compare peak heights with working standards for quantitative measurement. If peak height of sample solution is too large and extends off scale, dilute solution to appropriate volume.

Results and Discussion

One of the problems encountered in GLC or HPLC methods for the determination of 1080 is the difficulty in separating fluoroacetic acid from water that is used as the extraction solvent or that is co-extracted from tissue samples. Organic solvents used to partition fluoroacetic acid from water also extract an appreciable amount of water which later interferes in the various derivatization reactions used to convert fluoroacetic acid to a compound suitable for chromatographic analysis. The usual separation procedure is to evaporate the solvent and water after the addition of an inorganic base such as sodium or potassium hydroxide to form the fluoroacetate salt. This procedure requires critical adjustments of pH to prevent loss of fluoroacetic acid through defluorination (4) or conversion to glycolic acid (8). Separation of fluoroacetic acid by this procedure and later derivatization with PFBB resulted in low recoveries even with careful control of pH. Good recoveries, without pH adjustments, were obtained when triethanolamine was used as the base. Recoveries averaged 101 ± 4% (N = 4) and 96 ± 1% (N = 4), respectively, when 2 and 10 μg 1080 were extracted from water, taken through the concentration procedure, and derivatized with PFBB.

All solvents used were tested through the an-

alytical procedure to verify the absence of interfering contaminants. This was necessary when some solvents were found to contain impurities that appeared, either before or after derivatization, as extraneous peaks near the retention time of the 1080 derivative peak in the GLC determination. No problems due to impurities in the PFBB reagent were encountered in the GLC analysis despite the use of several different vials of the reagent purchased at different times. Chromatographic peaks due to compounds other than PFBB were present in the chromatograms but none was observed near the retention time of the 1080 peak. The PFBB reagent and 10% solution were stored at −10°C to minimize deterioration when not in use, and were brought to room temperature before opening for use to avoid condensation of moisture. The addition of a sufficient amount of PFBB was necessary to ensure complete derivatization of fluoroacetic acid in samples. Best results were obtained with 75–100 μL of the 10% PFBB solution. Smaller amounts occasionally resulted in low recoveries and larger amounts resulted in elevated baselines which made quantitative measurements more difficult for low concentrations of 1080.

The 3 gas chromatographic columns described under the method were used to obtain recovery data. In our work, the QF-1 and mixed QF-1/DC-200 columns were routinely used and the Ultrabond 20M was used as a backup column. Quantitative measurements were made on the 3 columns and percent recovery was usually calculated from the average of the 3 results which were generally within 8% of the mean. Occasionally, the controls of some stomach samples that were decayed or spoiled showed a peak near the retention time of 1080 on the QF-1 column but not on the QF-1/DC-200 or Ultrabond 20M columns. For these samples, the percent recoveries of added 1080 were calculated from the results obtained on the latter columns. Figure 1 shows chromatograms obtained with the 3 columns for the analysis of a 1 g sample of stomach fortified with 1 μg 1080. Not shown in the chromatograms are large, late-emerging peaks that varied in number and size with different samples. To shorten GLC analysis time, the oven temperature can be elevated to 200°C after the 1080 peak has appeared, to facilitate faster elution of the late-emerging compounds.

Recoveries of 1080 from 1 g samples of coyote stomach and muscle tissue fortified with 1080 averaged 50–58% at 0.1 ppm and 80–98% at 0.2–100 ppm (Table 1). Higher average recoveries of 90% were obtained at 0.1 ppm when 10 g samples of muscle tissue were fortified and a 10% aliquot of the sample extract was processed for cleanup and analysis. Although the lowest fortified level reported is 0.1 ppm, lower concentrations can probably be detected. Under typical operating conditions of the gas chromatograph, the derivative of 5–10 pg 1080 was easily detected at a signal-to-noise ratio of at least 2. If this detection limit is assumed for samples, a sensitivity of about 0.03–0.05 ppm is indicated. The determination of 0.1 ppm or less of 1080 may often be necessary for diagnosing 1080 poisoning, especially when canine tissues are being analyzed. We have detected residue levels near 0.1 ppm in the muscle tissue of a coyote that had orally ingested a lethal dose of 0.16 mg 1080/kg body weight (Table 2). Since the LD_{50} for canines is near 0.1 mg/kg, residue levels would be extremely low in tissues of an animal that consumed a minimum lethal dose of 1080.

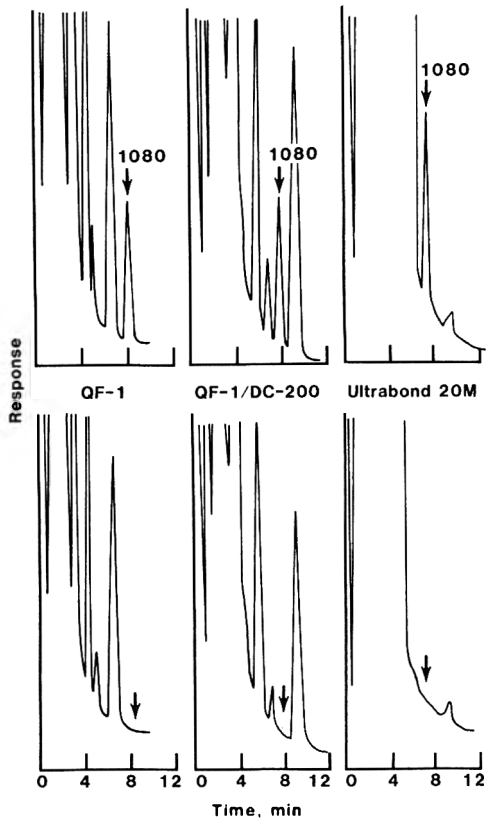


Figure 1. Gas chromatographic analysis of stomach content on 3 different colunns; lower chromatograms, control sample; upper chromatograms, 1 g sample fortified with 1 μg 1080; 1 μL injection (equivalent to 0.2 mg sample); attenuation at 64.

Table 1. Recovery of 1080 from 1 g samples of fortified tissue

Added, μg/g	Recoveries, %	Mean rec., % (± SD)
	Stomach	
0.1	56, 58, 49, 68	58 (±8)
0.2	77, 91, 85, 91	86 (±7)
1.0	105, 102, 81, 103	98 (±11)
10	95, 83, 94, 96	92 (±6)
100	89, 91, 99, 92	93 (±4)
	Muscle	
0.1	57, 38, 53, 52	50 (±8)
0.1[a]	80, 90, 100, 91	90 (±8)
0.2	94, 91, 101, 80	92 (±9)
1.0	99, 85, 90, 89	91 (±6)
10	81, 82, 80, 76	80 (±3)
100	85, 82, 81, 87	84 (±3)

[a] A 10 g sample was fortified and a 10% aliquot of extract solution was analyzed.

Table 2. Analysis of muscle tissues from 1080-poisoned coyotes

Dose, mg/kg	1080 found, ppm	Mean, ppm ± SD
0.16	0.12, 0.13, 0.11, 0.09	0.11 ± 0.02
0.23	0.08, 0.08, 0.07, 0.09	0.08 ± 0.01
0.50	0.14, 0.12, 0.15, 0.18	0.15 ± 0.02
5.0	1.9, 1.6, 1.8, 1.8	1.8 ± 0.13

All samples should be routinely analyzed on at least 2 dissimilar gas chromatographic columns and the 1080 peak should be identified by retention time on both columns to minimize false positive results. Our experience has indicated that the Ultrabond 20M and mixed QF-1/DC-200 columns are the most reliable. If necessary, additional confirmation can be made by GLC–mass spectrometry. Samples should be derivatized in a smaller volume of acetone to facilitate detection by mass spectrometry. The volume of acetone would depend on the amount of 1080 in the sample and the sensitivity of the mass spectrometer. The mass spectrum of pentafluorobenzyl fluoroacetate has been reported previously (6).

References

(1) Sawyer, R., Cox, B. G., Dixon, E. J., & Thomsen, J. (1967) *J. Sci. Food Agric.* **18,** 287–289
(2) Stahr, H. M., Buck, W. B., & Ross, P. L. (1974) *J. Assoc. Off. Anal. Chem.* **57,** 405–407
(3) Stahr, H. M. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1434–1435
(4) Peterson, J. E. (1975) *Bull. Environ. Contam. Toxicol.* **13,** 751–757
(5) Stevens, H. M., Moffat, A. C., & Drayton, J. T. (1976) *Forensic Sci.* **8,** 131–137
(6) Okuno, I., & Meeker, D. L. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 49–55
(7) Ray, C. R., Post, L. O., & Reagor, J. C. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 19–24
(8) Collins, D. M., Fawcett, J. P., & Rammell, C. G. (1981) *Bull. Environ. Contam. Toxicol.* **26,** 669–673

Gas-Liquid Chromatographic Determination of Fenvalerate Insecticide Residues in Processed Apple Products and By-Products

TERRY D. SPITTLER, ROBERT J. ARGAUER,[1] DONALD J. LISK,[2] RALPH O. MUMMA,[3] and GEORGE WINNETT[4]
New York State Agricultural Experiment Station, Pesticide Residue Laboratory, Cornell University, Geneva, NY 14456

Collaborators: E. Bogus; E. Greco; W. Gutenmann; R. Marafioti; E. Miles; P. Wszolek; L. Zimmerman

Apples from trees treated in the field at 2-week intervals (9 foliar applications) with the synthetic pyrethroid insecticide fenvalerate (cyano(3-phenoxyphenyl)methyl 4-chloro-alpha-(1-methylethyl)-benzeneacetate) were processed into apple sauce, juice, pomace, and peels plus cores. Gas-liquid chromatographic analysis of the commodities for fenvalerate showed the sauce and juice to be essentially residue-free, the whole apples to contain about 0.4 ppm, and the pomace and peels plus cores to contain about 2 and 1.5 ppm, respectively. Agreement among 5 laboratories using modifications of the same basic method was good.

Fenvalerate (cyano(3-phenoxyphenyl)methyl 4-chloro-alpha-(1-methylethyl)benzeneacetate), also known as Pydrin or Sumicidin, is an effective broad spectrum synthetic pyrethroid insecticide with adequate stability in the field. This compound is presently being used extensively on a variety of crops including fruits, field crops, and ornamentals. It is effective on pests resistant to other chemicals, has a low mammalian toxicity, and is used at low dosage rates compared with other agricultural chemicals (1). Although some residue information has been gathered on field-harvested crops, and all required toxicological and registration data have been submitted to and accepted by the Environmental Protection Agency (EPA Reg. No. 201-401), the overall data base is small, particularly with regard to processed commodities.

In the work reported, residues of fenvalerate were determined in field-treated apples, juice, canned sauce, pomace, and peels plus cores by participants in Northeast Regional Project No. 115 (NE-115).

The program provided an excellent opportunity for the 5 cooperating laboratories to compare data generated either by strict adherence to the manufacturer's method, or by modifying the method through a change in solvents, procedures, or equipment that a given laboratory might routinely use as a matter of preference, convenience, or necessity.

Cooperating laboratories are designated I-V. All sample production, processing, and distribution was done under the direction of Laboratory I.

Experimental

Samples

Apples.—Mature McIntosh and Red Rome apple trees in Orchard 12A at the New York State Agricultural Experiment Station (NYSAES) are arranged in random blocks with 4 other varieties. Sets of 3 replicate blocks were treated with the commercial formulation, Pydrin 2.4E (2.4 lb ai/gal. of emulsifiable concentrate), at the following rates: one set at the recommended rate (1×) of 2 oz ai/100 gal.; one set at twice the recommended rate (2×), i.e., 4 oz ai/100 gal.; one check (CH) set receiving no treatment.

Starting at petal fall, trees were sprayed to runoff with a hand sprayer at 460 psi on May 18, May 29, June 13, June 26, July 9, July 27, and August 7, 1979 for a total of 7 applications in the post-bloom control program. Two pre-bloom applications were applied on April 24 (half-inch green) and May 8 (pink).

One standard apple box (approx. 40 lb) of fruit was picked from each replicate tree and the 3 boxes for each set were composited and subdivided for processing. McIntosh apples were picked the second week in September 1979; the later-maturing Red Romes were harvested the second week in October 1979. Produce was held

[1] U.S. Dept of Agriculture, Agricultural Environmental Quality Institute, Beltsville, MD 20705.
[2] Cornell University, Toxic Chemicals Laboratory, Ithaca, NY 14853.
[3] Pennsylvania State University, Pesticide Laboratory, University Park, PA 16802.
[4] Rutgers University, Dept of Entomology and Economic Zoology, New Brunswick, NJ 08903.

Received October 28, 1981. Accepted February 11, 1982.
Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 3449.

0004-5756/82/6505-1106-06$01.00

Table 1. Production of apple juice and pomace

Sample	Mash yield,[a] kg/%	Juice yield,[b] kg/%	Pomace yield,[b] kg/%	Juice Brix[c]
RPCH	15.60/98	11.54/77	3.08/21	10.8
RPO1	15.60/98	11.80/79	2.70/18	12.2
RPO2	15.76/99	11.30/75	3.10/21	12.2
MPCH	15.83/99	12.92/86	2.52/17	11.3
MPO1	15.36/96	11.74/78	2.45/16	10.7
MPO2	15.56/97	11.99/80	2.70/18	12.0

[a] From 16.0 kg whole fruit, milled at 4200 rpm with No. 4 (1/4 in.) screen.
[b] From 15.0 kg mash, pressed 5 min at 500 psi plus 5 min at 1000 psi.
[c] % Sugar (dissolved solids).

in raw products storage (commercial conditions) at 35°C until processing in January 1980.

Whole fruit.—Approximately 10 kg apples from each variety-treatment composite were chopped in a Hobart food chopper. Care was taken to minimize the amount of juice formed as a result of chopping. After thorough mixing to ensure homogeneity, 1 kg subsamples were bagged and frozen (−20°C).

Apple juice and apple pomace.—Sixteen kg apples from each composite sample were milled on a Model D comminuting machine (W. J. Fitzpatrick Co., Chicago, IL) at 4200 rpm with a No. 4 screen to yield between 15.36 and 15.83 kg mash. See Table 1 for complete juicing parameters and yields. Exactly 15.00 kg mash from each batch was pressed on a small press of NYSAES design at 500 psi followed by 1000 psi. Juice yields were between 75 and 86% with pomace yields ranging between 21 and 16%. Juice subsamples of 1.8 L were frozen at −20°C in plastic bottles. Pomace subsamples of about 1.5 kg were bagged and frozen.

Applesauce and peels plus cores.—Approximately 100 apples from each composite set were automatically peeled and cored on a Pease 4-station apple peeler and deposited in an air-agitated cold water bath. Collected peels and cores comprised between 19.8 and 25.5% of the starting weights (see Table 2 for saucing details). An additional 0.7–2.2% of the fruit weight was hand-trimmed before dicing. Apples were conveyed to a Model B dicer (Urschel Laboratories, Valparaiso, IN), where they were sliced lengthwise into $3/4 \times 5/11$ in. pieces suitable for pulping in a Langseenamp laboratory pulper-finisher at 800 rpm with a 1/8 in. mesh screen. Sauce was cooked in a continuous-feed cooker of NYSAES design at 207–212°F. The consistencies and the percent solids of the sauces were measured, and water and sugar were added as necessary to produce products of commercially acceptable properties. Sauces were then automatically measured and sealed in No. 303 (15 1/2 oz) cans on an American Can Co. 34-A 006 vacuum closing machine. Canned sauce was stored at room temperature until distribution. Peels and cores plus small amounts of trimming and pulping waste were mixed and divided into 500 g subsamples which were held at −20°C.

Identical sets of 30 samples were distributed to the 5 participating laboratories. Each set consisted of Pydrin-treated samples at 1×, 2×, and check rates for both McIntosh and Red Romes—coded MPO1, MPO2, MPCH, RPO1, RPO2, and RPCH, respectively. Each coded

Table 2. Production of applesauce and peels and cores

Sample	Wt to peeler, kg	Waste,[a] kg/%	Sol. solids, %	Sugar added, %	Water added, %	Consistency[b] before/after
RPCH	16.19	3.95/24.4	8.0	8.2	29.0	3.0/4.8
RPO1	18.62	4.78/25.7	8.2	8.0	30.3	1.9/4.6
RPO2	15.74	4.01/25.5	9.2	6.2	14.0	3.6/4.4
MPCH	15.18	4.36/28.7	9.7	6.2	10.9	3.6/4.4
MPO1	15.82	4.57/28.9	9.7	6.2	0.0	4.3/4.3
MPO2	16.25	4.52/27.8	9.6	6.4	8.5	3.0/4.5

[a] Peels, cores, trimmings, and pulper/finisher waste.
[b] cm/s before and after adjustment with sugar and water.

sub-set contained one sample each of chopped whole fruit, apple juice, apple pomace, peels plus cores, and canned applesauce.

Copies of the residue method and fenvalerate analytical standards (99%) were provided courtesy of the manufacturer, Shell Development Co., Biological Sciences Research Center, Modesto, CA 95352 (2).

Methods

Standard laboratory glassware and pesticide grade solvents were used by all laboratories. Blenders and equipment commonly found in any residue laboratory were used. Unique methods and equipment for each laboratory are described.

Extraction and Cleanup of Samples

Laboratory I.—Blend 50 g samples with 100 mL acetone for 3 min, filter, and transfer filtrate to 500 mL separatory funnel. Add 30 mL saturated Na_2SO_4 solution, 100 mL water, and 100 mL Skelly-Solve B. Shake 1 min, let phases separate, and draw off and discard bottom aqueous layer. Force 10 mL aliquot of organic layer through Florisil Sep-Pak cartridge (Waters Associates) and collect and save eluate for gas-liquid chromatography (GLC).

Laboratory II.—Weigh 50 g sample into Sorvall Omni-Mixer cup (500 mL size) with 200 mL hexane–isopropanol (3 + 1). Blend at high speed 2 min with cup lowered in ice-water bath and filter through paper into 250 mL separatory funnel. After rinsing blender cup and filter 2 times with 20 mL solvent (hexane–isopropanol), remove isopropanol by adding 100 mL water and shaking carefully for 1 min; drain and then discard lower (aqueous) phase. Wash hexane layer with 2 additional 100 mL volumes of water to remove all isopropanol, drain into 200 mL beaker, and add boiling chip. Reduce volume on steam table to concentration suitable for injections for GLC (50–100 μL). Where necessary, further concentrations are carried out under a stream of nitrogen.

Laboratory III.—The whole fruit, cores plus skins, pomace, and sauce are analyzed as follows: Weigh ca 50 g sample into Waring blender equipped with 1 qt screw-cap glass jar. Add 30 g Na_2SO_4 and 200 mL methylene chloride and blend 2 min. Filter supernate through Whatman 2V paper and transfer one-half the total volume (100 mL) to 250 mL round-bottom flask. Evaporate methylene chloride in rotary evaporator at 42°C under vacuum until just dry. Redissolve residue in 20 mL hexane; transfer to foil-lined screw-cap vial for GLC analysis.

Treat the juice differently: Transfer 50 g juice to 500 mL separatory funnel and add 50 mL saturated NaCl solution and 200 mL methylene chloride. Shake separatory funnel 2 min and let layers separate. Filter methylene chloride through Na_2SO_4 pad, transfer aliquot of one-half total volume (100 mL) to round-bottom flask, and evaporate in rotary evaporator at 42°C until just dry. Redissolve juice residue in 20 mL hexane and transfer to foil-lined screw-cap vial.

Laboratory IV.—Blend 50 g sample 3 min with 150 mL hexane–isopropanol (3 + 1). Filter; then wash 90 mL aliquot in separatory funnel 3 times with 100 mL water. Elute hexane fraction through 6 g Florisil column which is then washed with additional 50 mL hexane. Elute fenvalerate with 50 mL ethyl acetate–hexane (5 + 95) and concentrate to 1.0 mL for GLC.

Laboratory V.—Applesauce: Blend 100 g sample with 200 mL methylene chloride, and filter. Concentrate 50 mL aliquot to near dryness on Rinco evaporator, and dissolve residue in 10 mL hexane for GLC.

Table 3. Fenvalerate residues in chopped whole apples (ppm)

Lab.[a]	RPCH	RPO1	RPO2	MPCH	MPO1	MPO2	Recovery, %
I	<0.25	0.46	0.41	<0.25	0.25	0.30	100 at 0.4 ppm
II	<0.05	0.28	0.44	<0.05	0.32	0.49	116 at 0.18 ppm
III	<0.01	0.31	0.95	<0.01	0.36	1.07	97 at 2.0 ppm
IV	<0.16	0.34	0.53	<0.12	0.57	0.51	110[b]
V	<0.01	0.63	0.60	<0.01	0.45	0.60	90–110 at 0.01 ppm
$\bar{X}$		0.40	0.58		0.38	0.59	
S		±0.14	±0.22		±0.14	±0.59	

[a] I = Cornell University, Geneva, NY
II = Cornell University, Ithaca, NY
III = Pennsylvania State University, University Park, PA
IV = Rutgers University, New Brunswick, NJ
V = U.S. Dept of Agriculture, Beltsville, MD.

[b] Average of recoveries run at 0.1, 0.5, 1, and 2 ppm.

Table 4. Fenvalerate residues in apple juice (ppm)

Lab.[a]	RPCH	RPO1	RPO2	MPCH	MPO1	MPO2	Recovery, %
I	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	98 at 0.3 ppm
II	<0.005	<0.005	<0.005	<0.01	<0.01	<0.01	100 at 0.0036 ppm
III	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	83 at 2.0 ppm
IV	<0.15	<0.15	<0.14	<0.18	<0.18	<0.13	101[b]
V	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	90–110 at 0.01 ppm

[a,b] See footnotes, Table 3.

Apple juice: Shake 100 g sample with 200 mL methylene chloride in separatory funnel. Concentrate 50 mL aliquot of methylene chloride layer to near dryness on Rinco evaporator, and dissolve residue in 10 mL hexane for GLC.

Apple skins and cores: Blend 100 g sample with 200 mL methylene chloride, and filter. Transfer 2 mL aliquot to glass vial, concentrate to near dryness with nitrogen purge, and dilute with 15 mL hexane for GLC.

Whole fruit: Blend 100 g sample with 200 mL methylene chloride. Add anhydrous Na_2SO_4 to break emulsion. Filter sample, and concentrate 5 mL aliquot to near dryness with nitrogen purge; dilute with 50 mL hexane for GLC.

Pomace: Blend 50 g of sample with 200 mL methylene chloride, filter, and concentrate 1.0 mL filtrate to near dryness with nitrogen purge. Dilute with 50 mL hexane for GLC.

Chromatographic Analyses

Laboratory I.—Analyze sample on Tracor Model 220 gas chromatograph equipped with ^{63}Ni electron capture detector, using 3 ft × 1/4 in. glass column of 5% OV-101 on 80–100 mesh Gas-Chrom Q, and the following conditions: column 237°C, detector 295°C, inlet 220°C, nitrogen carrier gas. Samples will chromatograph as single peaks; compare areas with those for standards on a 4-point standard curve.

Laboratory II.—Analyze samples on Tracor Model 222 gas chromatograph equipped with ^{63}Ni detector and 180 cm × 4 mm id glass column of 3% OV-17 on 100–120 mesh Gas-Chrom Q, and using the following conditions: column 245°C, detector 295°C, inlet 270°C, nitrogen carrier gas. Fenvalerate is resolved as 2 peaks with retention times of 16.9 and 18.8 min. Combine peak areas for each sample and compare with those on a standard curve.

Laboratory III.—Analyze samples on Tracor Model 222 gas chromatograph equipped with ^{63}Ni detector and 4 ft × 4 mm id column of 3% SP-2100 on 80–100 mesh Supelcoport, and using the following conditions: column 240°C, detector 310°C, inlet 250°C, nitrogen carrier gas. Quantitate fenvalerate by comparing area of first peak (340 s retention time) with areas obtained with standards for this peak. Retention time of second peak is 370 s.

Laboratory IV.—Analyze samples on Tracor Model 220 gas chromatograph equipped with thermionic (N-P) detector and 4 ft × 2 mm id glass column of 3% Dexsil 300 on 100–120 mesh Supelcoport, and the following conditions: column 250°C, detector 275°C, helium carrier gas. Integrate both peaks and compare independently with corresponding peaks derived from standard (retention times 4.0 and 4.5 min). Report residues as average of the 2 values.

Laboratory V.—Analyze sample on Tracor Model 550 gas chromatograph equipped with ^{63}Ni electron capture detector and 6 ft × 4 mm id column of 3% SP-2100 on 100–120 mesh Supelcoport, and the following conditions: column 260°C, detector 300°C, inlet 260°C. Inject 5 μL

Table 5. Fenvalerate residues in apple pomace (ppm)

Lab.[a]	RPCH	RPO1	RPO2	MPCH	MPO1	MPO2	Recovery, %
I	<0.25	0.75	1.5	<0.25	0.9	0.9	95 at 0.4 ppm
II	<0.1	1.2	1.8	<0.1	1.6	1.8	85 at 0.36 ppm
III	<0.01	2.09	3.61	<0.01	2.29	3.69	83 at 0.5 ppm
IV	trace	1.34	1.33	trace	1.28	2.25	109[b]
V	<0.01	2.7	3.3	<0.01	4.0	4.0	90–100 at 0.01 ppm
$\bar{X}$		1.6	2.3		2.0	2.5	
S		±0.77	±1.1		±1.2	±1.3	

[a,b] See footnotes, Table 3.

Table 6. Fenvalerate residues in applesauce

Lab.[a]	RPCH	RPO1	RPO2	MPCH	MPO1	MPO2	Recovery, %
I	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	93 at 0.6 ppm
II	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	104 at 0.36 ppm
III	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	91 at 2.0 ppm
IV	<0.07	<0.06	<0.06	<0.06	<0.08	<0.07	100[b]
V	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	90–110 at 0.01 ppm

[a,b] See footnotes, Table 3.

aliquots, measure peak heights for both isomers and compare with those obtained for 100 and 500 pg standard injections in 5 μL hexane.

Results and Discussion

The processing parameters employed and the yields of products obtained (Tables 1 and 2) are similar to commercial production figures. Peels plus cores are frequently diverted to a juice production line, but it was instructive in this study to analyze them separately.

The concentrations of fenvalerate residue found in the whole apples, products, and by-products are given in Tables 3–7. The data appear to be in good agreement among the 5 laboratories. This verifies that the basic analytical method, even though used with modifications by each laboratory, is sound. The data indicate that fenvalerate is absent in apple juice or sauce but is associated with the pomace and peels plus cores, and is also found in the fresh fruit. This implies that the residues adhere to the skin of the fruit and presumably remain with it during subsequent processing.

It is interesting to note that the residues found in the whole apple can be accounted for in the analyses of its processed fractions. For instance, for the McIntosh apples coded MPO1, the average residue found was 0.38 ppm (Table 3). If one takes the average yield of pomace obtained from the fruit (18.5%) and multiplies by the average residue concentration of fenvalerate found in the MPO1 pomace which is 2.0 ppm (Table 5), the value is 0.37 ppm. Similarly, if one uses the average yield of peels plus cores obtained from the apples (26.8%) and multiplies by the average concentrations of fenvalerate found in them for MPO1, which is 1.5 ppm (Table 7), the calculated value is 0.40 ppm. The concentrations of insecticide in the juice or sauce do not enter into the calculations because they were essentially nil.

In an earlier study by Wszolek et al. (3), about 0.5% of the dose of fenvalerate fed to a lactating cow was excreted intact in the milk. Apple pomace has been used in the past in dairy cattle rations, so the possible concentration of fenvalerate residues in the pomace fraction would have to be considered. Because apple sauce and juice appear to be free of fenvalerate residues, its use on apples destined for baby food production should pose a minimal health hazard.

Acknowledgments

The authors thank the following personnel for participating in the collaborative study:

E. Bogus, Pennsylvania State University, Pesticide Laboratory, University Park, PA

E. Greco and R. Marafioti, State Agricultural Experiment Station, Geneva, NY

W. Gutenmann and P. Wszolek, Cornell University, Toxic Chemicals Laboratory, Ithaca, NY

E. Miles, U.S. Dept of Agriculture, Agricultural Environmental Quality Institute, Beltsville, MD

Table 7. Fenvalerate residues in apple peels and cores

Lab.[a]	RPCH	RPO1	RPO2	MPCH	MPO1	MPO2	Recovery, %
I	<0.25	1.2	1.7	<0.25	1.2	1.3	77 at 0.4 ppm
II	<0.1	0.7	2.1	<0.1	1.1	1.4	none reported
III	<0.01	1.84	4.10	<0.01	2.18	3.34	88 at 1.0 ppm
IV	trace	1.21	2.27	trace	1.36	1.95	77[b]
V	<0.01	1.4	2.9	<0.01	1.9	2.4	90–110 at 0.01 ppm
$\bar{X}$		1.3	2.6		1.5	2.1	
S		±0.4	±0.9		±0.5	±0.8	

[a,b] See footnotes, Table 3.

L. Zimmerman, Rutgers University, Dept of Entomology and Economic Zoology, New Brunswick, NJ

REFERENCES

(1) *Farm Chemicals Handbook* (1981) Meister Publishing Co., Willoughby, OH, p. C148
(2) Determination of SD 43775 Residues in Crops, Animal Tissues, Soil, and Water (1978) Modesto Method Series MMS-R-478-1, Shell Development Co., Modesto, CA
(3) Wszolek, P. C., Lein, D. H., & Lisk, D. J. (1980) *Bull. Environ. Contam. Toxicol.* 24, 296–298

Influence of Various Solvent-Water Mixtures on the Extraction of Dieldrin and Methomyl Residues from Radishes

WILLIS B. WHEELER, NEAL P. THOMPSON, ROBERT L. EDELSTEIN, RAMON C. LITTELL,[1] and RICHARD T. KRAUSE[2]
University of Florida, IFAS, Pesticide Research Laboratory, Gainesville, FL 32611

The effect of organic solvent/water ratios on the extraction of field-incurred residues of dieldrin and methomyl from radishes was determined. ^{14}C-Dieldrin and ^{14}C-methomyl were applied separately to radishes in commercial formulations at rates of 0.2 and 0.9 kg/ha, respectively. Fourteen days post-application, the radishes were harvested and fresh root tissues were extracted using a Polytron homogenizer. Acetone, acetonitrile, and methanol containing 0, 10, 20, 30, 40, and 50% water were used as extraction solvents. For methomyl residues, the optimum water content of acetonitrile-water extraction mixtures was 40-50%; less than 40% water reduced the ability of acetonitrile to extract carbon-14. Methanol and acetone were nearly as efficient as 50% acetonitrile-water and were apparently not influenced by solvent/water ratios. For dieldrin, low water content slightly reduced the extraction efficiency of acetonitrile, with the optimum water content also being 40-50%. Percentage of water appeared to have little overall effect on methanol extraction efficiency. The extraction efficiency of acetone was lower than that of the other 2 solvents, and this effect was independent of the acetone/water ratio. Approximately 20% of the ^{14}C-dieldrin residue was bound to radish roots 14 days post-application.

Since the advent of synthetic organic pesticides, a variety of techniques and solvents have been used to extract these residues before analytical determination. During the 1940s and into the mid-1950s, nonpolar solvents were used to extract organic pesticides from plant materials (1). This procedure often led to erratic recoveries of pesticides added to untreated check samples. In the latter part of the 1950s, Klein (2) and Lichtenstein (3) reported increased extraction efficiency when mixtures of polar and nonpolar solvents were used. By the early 1960s, Mills et al. (4) demonstrated the effectiveness of using a single polar solvent, acetonitrile, to extract chlorinated pesticide residues from low fat substrates. Bertuzzi et al. (5) subsequently demonstrated that acetonitrile alone did not extract all the field-incurred residues present in low moisture content plant material. It was further demonstrated and later confirmed by Wilderman and Shuman (6) and Burke et al. (7) that the use of 35% water in acetonitrile resulted in improved extraction of residues from low moisture plant material; residue levels determined compared favorably with levels found using the exhaustive process of Mumma et al. (8) and Wheeler et al. (9). Little, if any, work has been reported since Bertuzzi et al. (5) evaluated various solvent-water combinations as extracting solvents. The purpose of the study reported here was to determine the effect of various solvent/water ratios on the extraction of field-incurred residues of a nonpolar pesticide or polar pesticide from high moisture crops. Acetone, acetonitrile, and methanol containing from 0 to 50% water were selected for study. The high moisture crop chosen was radishes because previous studies had shown that field-incurred pesticide residues were difficult to extract from this root vegetable (10-12). Dieldrin (1,2,3,4,10,10-hexachloro-*exo*-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*,*exo*-5,8-dimethanonaphthalene) and methomyl [*S*-methyl *N*-(methylcarbamoyl)oxythioacetimidate], nonpolar and polar pesticides, respectively, were selected for this study.

Experimental

Radishes (red globe variety) were germinated in flats of soil and later transplanted into 15 cm plastic pots. Plants were maintained in a greenhouse until treatment and thereafter in a controlled environmental chamber (Scherer-Gillet Model CEL-512-37, Troy, MI 48084). Periods of light and dark were 12 h each. Temperatures were 24°C (light) and 20°C (dark). Water, fertilizer, and pest control measures were applied as needed.

Ring-^{14}C-labeled dieldrin (specific activity 85 mCi/mM, Amersham Corp., Arlington Heights, IL 60005) and *S*-methyl-^{14}C-labeled methomyl (specific activity 3.5 mCi/mM, New England Nuclear Corp., Boston, MA 02118) were shown

Florida Agricultural Experiment Station Journal Series No. 3062.

Mention of a pesticide or commercial product does not constitute a recommendation or endorsement of this product by the U. S. Department of Health and Human Services, Food and Drug Administration (FDA), or the University of Florida.

This study was supported by FDA contract 223-76-2220.

[1] University of Florida, IFAS, Statistics Department.
[2] Food and Drug Administration, Division of Chemical Technology, Washington, DC 20204.

Received December 1, 1981. Accepted March 2, 1982.

0004-5756/82/6505-1112-06$01.00

to be 98+% pure by thin layer chromatography (TLC) before use. The ^{14}C-labeled insecticides were mixed with commercial formulations of the same compound (18.7% dieldrin formulation, Shell Chemical Co., New York, NY 10022; 90% Lannate formulation, du Pont de Nemours & Co., Inc., Wilmington, DE 19898) to give application rates of 0.2 and 0.9 kg/ha, respectively, for dieldrin and methomyl. Plants were placed in bacteriological-type hoods and chemicals were sprayed onto the radish root tops and surrounding soil, using an aerosol can-powered device similar to those used for spraying chromogenic reagents on TLC plates.

Treated plants were placed in environmental chambers; after 14 days, the radishes were pulled from the soil, the tops were cut off and discarded, and the roots were rinsed with water to remove adhering soil. The roots were chopped (Model 84141, Hobart Manufacturing Co., Troy, OH 45374) and mixed and then 100 g aliquots were weighed into 32 oz glass jars (Tropicana Products Inc., Bradenton, FL 33506).

Three 100 g samples were extracted with methanol and then 3 each with 10, 20, 30, 40, and 50% water in methanol (18 samples). The procedure was repeated using acetone and acetonitrile and the same water-solvent mixtures. All other steps of the extraction process, described below, were held constant for all samples.

Each 100 g portion of the chopped radish roots was blended with 200 mL solvent, using a Polytron ultrasonic homogenizer (Model PT-10-35 with a PT 35 K generator) at high speed for 1.0 min. Each blended sample was vacuum-filtered through a 60 mm id coarse fritted glass funnel. The Polytron blades and blender jar were rinsed with 50 mL solvent. The wash was transferred to a filter funnel and passed through a filter, using vacuum. The wash solution was combined with the original filtrate to make Fraction 1. Two hundred mL solvent was added to the blender vessel, blended 10 s, and transferred to the original filter funnel, and crop material was leached at 5 mL/min to make Fraction 2. Crop material was then leached with three 100 mL portions of solvent, each collected individually to yield Fractions 3, 4, and 5.

Each of the tissue samples remaining from the above blend-leach process was extracted with chloroform–methanol (9 + 1) in a Soxhlet extractor for 16 h (Fraction S).

Each fraction and the tissue residue which remained after Soxhlet extraction were analyzed in duplicate for radioactivity. All samples were combusted in a liquid scintillation sample oxidizer (Teledyne Intertechnique IN4101, Fairfield, NJ 07006).

To prepare the samples for combustion, aliquots of each fraction were pipetted into 7.6 × 7.6 cm squares of cellophane (Cello-Flex, Carolina Biological Supply Co., Burlington, NC 27215) and allowed to evaporate to dryness. The cellophane squares were then folded and placed in polycarbonate capsules designed for the sample oxidizer. In the case of tissue residues, small portions of the residual tissue were weighed directly into capsules.

Recoveries of ^{14}C-methomyl and ^{14}C-dieldrin added to extracts of radish roots and taken through the evaporation and combustion steps averaged 90 and 94%, respectively. No statistically significant correlations between total carbon-14 recovery and solvent/water ratios were found.

The sample oxidizer automatically added a premixed scintillation solution (phenethylamine–methanol–toluene–water–Permafluor, 33 + 22 + 35 + 5 + 4) to the counting vial. Each sample was then counted in a liquid scintillation spectrometer (Model 3375, Packard Instrument Co., Downers Grove, IL 60515). Samples were counted for 10 min or 10 000 counts, using the factory-set carbon-14 window and amplification. All sample counts were corrected for background counts but no corrections were made for quenching effects because automatic external standard ratios were similar for all samples. The total radioactivity in each fraction and the tissue residue was determined and percentages in each were calculated.

Linear regression of the data was performed using the General Linear Models procedure of the Statistical Analysis System (13). Percent carbon-14 values for Fractions 1 and 2 and for tissue residue were regressed on the percentage of water, using a linear model, and 95% confidence bands for the mean percent carbon-14 were obtained about the regression lines.

Selected Fraction 1 extracts were concentrated, and the aqueous portion remaining was partitioned with ethyl acetate (methomyl residues) or methylene chloride (dieldrin residues). Organic-soluble materials were spotted on silica gel TLC plates along with authentic samples of methomyl and dieldrin. TLC plates were developed in ethyl acetate for methomyl extracts or in ether–hexane (1 + 1) for dieldrin extracts.

Results and Discussion

The percentages of total carbon-14 extracted for dieldrin- and methomyl-treated radishes are

Table 1. Total percentage carbon-14 extracted 14 days post-treatment

Water, %	Acetone	Acetonitrile	Methanol
	Dieldrin		
0	73.6	74.9	79.7
10	73.1	77.2	79.4
20	75.5	79.6	80.1
30	76.8	79.4	80.6
40	76.5	81.1	81.1
50	76.1	81.7	81.7
	Methomyl		
0	64.4	54.8	66.0
10	62.9	62.1	63.7
20	64.5	65.1	62.6
30	64.6	64.9	67.6
40	63.2	66.9	65.9
50	66.2	67.2	64.7

presented in Table 1. This table summarizes the effects of various solvent-water combinations on extraction efficiencies.

The observed mean percentage carbon-14 values obtained for Fraction 1 (initial extract), Fraction 2 (200 mL jar and tissue rinse), and tissue residue (remaining extracted crop marc) are plotted in Figures 1–6. Also presented are the linear regression lines with the 95% confidence bands for each pesticide and solvent-water combination. Linear regression lines were used because the data generally fitted a straight line.

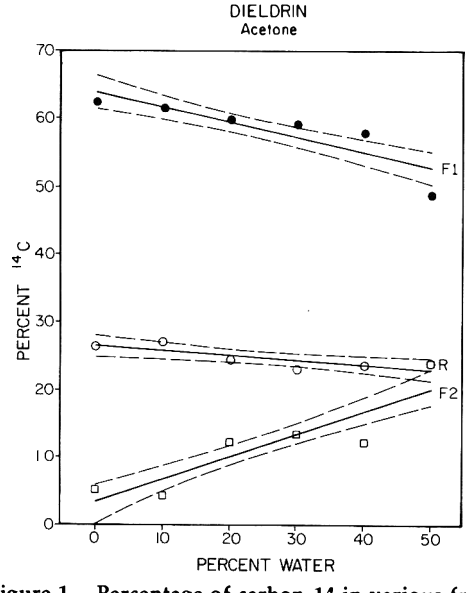


Figure 1. Percentage of carbon-14 in various fractions after extracting ^{14}C-dieldrin-treated radishes, using water-acetone mixtures. (Regression lines are solid lines, 95% confidence bands are dashed lines, and average of triplicate [observed mean] carbon-14 values are points. F1, F2, and R are Fractions 1, 2, and tissue residue, respectively, as defined in the text.)

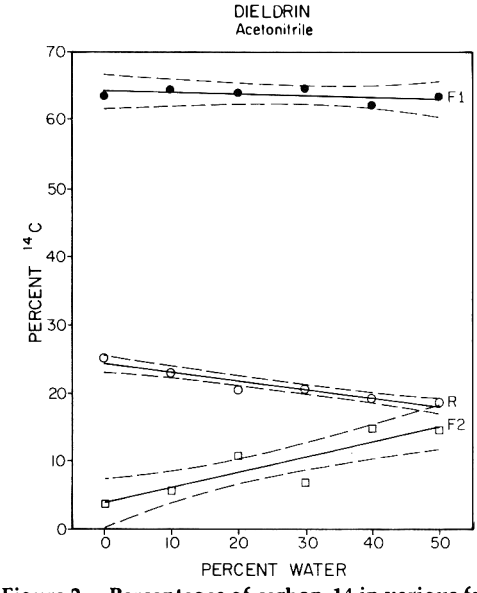


Figure 2. Percentages of carbon-14 in various fractions after extracting ^{14}C-dieldrin-treated radishes, using water-acetonitrile mixtures (see Fig. 1 for description of terms).

Dieldrin

Results for the extraction of dieldrin-treated radishes are presented in Figures 1–3.

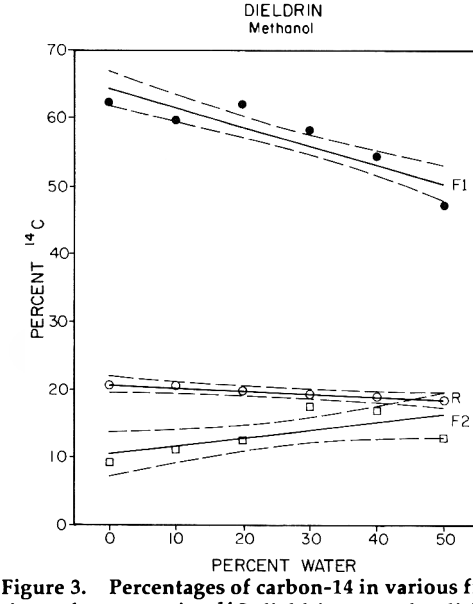


Figure 3. Percentages of carbon-14 in various fractions after extracting ^{14}C-dieldrin-treated radishes, using water-methanol mixtures (see Fig. 1 for description of terms).

Acetone.—Extraction of carbon-14 from radishes, using acetone-water mixtures, is shown in Figure 1. The percentage of carbon-14 in Fraction 1 decreased as the percentage water increased (64% with 0% water and 53% with 50% water). The values for Fraction 2 tended to increase as Fraction 1 values decreased. Carbon-14 in Fraction 2 was approximately 3% for 0% water and increased to 20% for 50% water in acetone. The carbon-14 content of the tissue residue decreased slightly from 27 to 23% (over the range from 0 to 50% water).

Acetonitrile.—Data showing the extraction of dieldrin-treated radishes, using acetonitrile-water, are presented in Figure 2. Carbon-14 found in Fraction 1 remained relatively constant (approximately 64%) for all water ratios. Fraction 2 carbon-14 values showed an overall increasing trend, ranging from 4 to 15%, as the percentage of water increased. The carbon-14 extracted with the second leach solution (Fraction 3, not shown) was relatively low and constant. Tissue residue values decreased consistently over the entire acetonitrile-water range. At 0% water, the tissue residue contained 24% of the carbon-14 and when 50% water-acetonitrile was used, 18%.

Methanol.—Figure 3 presents data for the extraction of dieldrin-treated radishes, using methanol-water mixtures. Fraction 1 values decreased substantially and sporadically from about 65% to about 50% extracted from 0 to 50% water. Fraction 2 carbon-14 values increased from 10% (at 0% water) to 17% (at 50% water). Fraction 3 values (not shown) were again low and relatively constant, although at 50% water Fraction 3 contained 6% of the total radioactivity; this represented the highest of any of the Fraction 3 values. Tissue residue levels remained constant at 18–20% regardless of the percentage of water.

In the case of dieldrin, acetonitrile-water mixtures did not alter the percentage of radioactivity present in Fraction 1, whereas with acetone-water and methanol-water, Fraction 1 values decreased significantly as the percentage of water increased. The insolubility of dieldrin in these aqueous solutions may have caused the Fraction 1 values to decrease as the water content increased. This hypothesis is supported by the increase of carbon-14 values in Fraction 2 when acetone and methanol are used as solvents. Methanol-water and acetonitrile-water mixtures were equally effective in removing total radioactivity as judged by that remaining in the tissue residue. A larger volume of extracting solvent was required when high percentages of water in methanol were used.

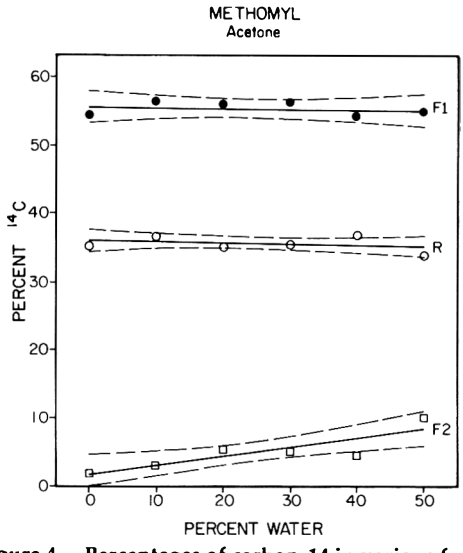


Figure 4. Percentages of carbon-14 in various fractions after extracting ^{14}C-methomyl-treated radishes, using water-acetone mixtures (see Fig. 1 for description of terms).

The highest overall extraction efficiency (lowest tissue residue value) is most desirable. Tissue residue values for acetone-water mixtures were in the 23–26% range and were significantly higher (1% confidence level) than for methanol and acetonitrile-water values, which were 18–19% under conditions of optimum extraction efficiency. Thus acetone (or acetone-water) did not extract dieldrin as efficiently as did acetonitrile- or methanol-water combinations.

The radioactivity extracted from dieldrin-treated radishes was determined by TLC to be 95% parent compound.

In radishes, 20% of the carbon-14 applied as dieldrin could not be recovered even when tissues were exhaustively extracted using chloroform–methanol in a Soxhlet extractor (8, 9).

Methomyl

Results for the extraction of methomyl-treated radishes are presented in Figures 4–6.

Acetone.—Extraction of carbon-14 from radishes, using acetone or acetone-water mixtures, is illustrated in Figure 4. In this case, the percentages detected in Fraction 1 (55%) and in the tissue residue (35%) remained nearly constant over the entire range of solvent-water ratios. Fraction 2 values increased with increasing water content except for random deviations (statistical residues) about the general trend. The range of

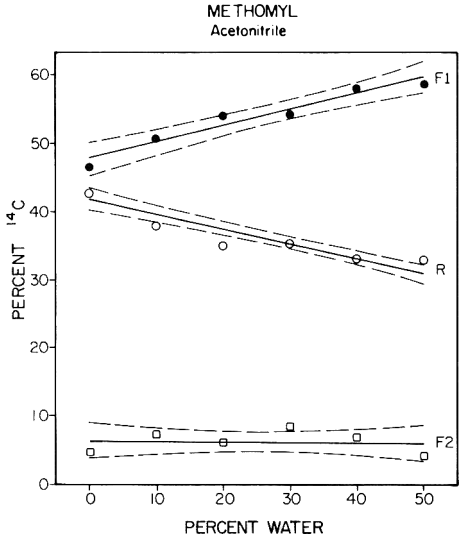


Figure 5. Percentages of carbon-14 in various fractions after extracting ^{14}C-methomyl-treated radishes, using water-acetonitrile mixtures (see Fig. 1 for description of terms).

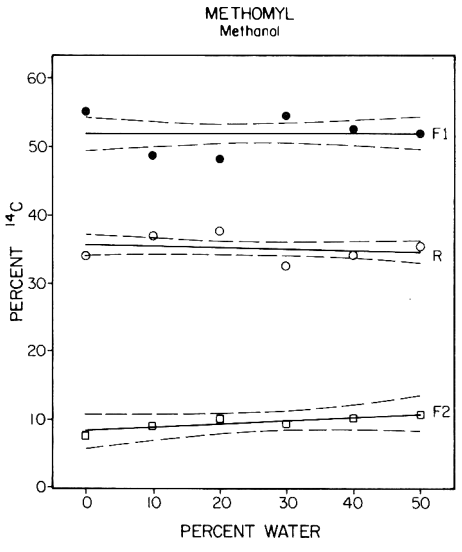


Figure 6. Percentages of carbon-14 in various fractions after extracting ^{14}C-methomyl-treated radishes, using water-methanol mixtures (see Fig. 1 for description of terms).

values for Fraction 2 was from 2 to 10%. Fraction 3 (not shown) was small, approximately 1% of the total radioactivity, over the entire range of acetone-water mixes.

Acetonitrile.—Figure 5 presents the results, using acetonitrile-water mixtures to extract methomyl-treated radishes. Two trends were apparent: (*1*) the percentage of radioactivity present in Fraction 1 increased with increasing water, and (2) the percentage of radioactivity remaining in the tissue residue decreased with increasing water. Fraction 2 remained relatively constant at about 7% over the entire range of solvent-water composition.

Methanol.—The results, using methanol-water mixtures to extract methomyl-treated radishes, are shown in Figure 6. Except for random variations, Fraction 1 was unaffected by the solvent/water ratios, and contained an average of 52% of the carbon-14 content. The tissue residue values representing approximately 35% of the radioactivity reflect the fluctuations of the Fraction 1 values; tissue residue statistical residuals increased as Fraction 1 statistical residuals decreased. Fraction 2 values increased slightly over the range of solvent-water mixtures, increasing from approximately 8% at 0% water to 11% at 50% water.

One factor was apparent from the extraction data generated from methomyl-treated radishes. The deviations of the observed Fraction 1 points from the Fraction 1 regression lines are negatively correlated with the corresponding tissue residue statistical residuals. Fraction 1 statistical residuals show smaller negative correlations with Fraction 2 statistical residuals. This indicates that if an unusually large (small) amount of radioactivity is extracted in Fraction 1 for a sample, then an unusually small (large) amount will tend to show up in the tissue residue, but the amount extracted in Fraction 2 will be little affected. This phenomenon is particularly exhibited in Figure 5 where the tissue residue values decrease while Fraction 1 values increase with increasing percent water, but Fraction 2 remains essentially constant. Thus if the carbon-14 was not extracted in Fraction 1, it would probably not occur in subsequent fractions.

Fraction 2 does contain a significant portion of the radioactivity, for in each case Fraction 2 possessed as much as 10% of the extractable carbon-14. The leaching process, starting immediately after the rinsing of blender blades, jar, and blended crop, removed only minor quantities. Subsequent fractions collected (Fractions 3, 4, 5, and S) possessed relatively insignificant amounts of radioactivity.

Although more carbon-14 was extracted from methomyl-treated radishes as the percentage water in acetonitrile-water extractant increased, there is no evidence (based on TLC) that increasing quantities of methomyl were extracted.

Fourteen days after application to radishes, only 10% of the extractable carbon-14 was intact methomyl.

Summary

The use of acetonitrile alone to extract pesticides from fresh plant materials should be evaluated on a broad basis. Although methomyl may not have been an ideal example of a polar insecticide because of its rapid metabolism, the data for both methomyl and dieldrin clearly indicate that the behavior of acetonitrile as an extracting solvent changes with water content, whereas this was not the case for acetone and methanol. The effectiveness of acetonitrile as an extracting solvent increased with increasing water content. Methanol needs additional evaluation as an extraction solvent; it appears to exhibit good extraction efficiency, which is not influenced by variable water content.

In previous work (10, 12) related to pesticide extraction efficiency, considerable variation had been noted among plant species. Thus, to evaluate this phenomenon more completely, the extraction of several chemicals from a variety of substrates should be studied.

Acknowledgments

The authors thank Nora Maddox, Laura Kennedy, and Cheryl Devore for technical assistance.

References

(1) Wheeler, W. B., & Frear, D. E. H. (1966) *Residue Rev.* **16,** 86–102

(2) Klein, A. K. (1958) *J. Assoc. Off. Agric. Chem.* **41,** 551–555

(3) Lichtenstein, E. P. (1959) *J. Agric. Food Chem.* **7,** 430–433

(4) Mills, P. A., Onley, J. H., & Gaither, R. A. (1963) *J. Assoc. Off. Agric. Chem.* **46,** 186–191

(5) Bertuzzi, P. F., Kamps, L., & Miles, C. I. (1967) *J. Assoc. Off. Anal. Chem.* **50,** 623–627

(6) Wilderman, M., & Shuman, H. (1968) *J. Assoc. Off. Anal. Chem.* **51,** 892–895

(7) Burke, J. A., Porter, M. L., & Young, S. J. V. (1971) *J. Assoc. Off. Anal. Chem.* **54,** 142–146

(8) Mumma, R. O., Wheeler, W. B., Frear, D. E. H., & Hamilton, R. H. (1966) *Science* **152,** 530

(9) Wheeler, W. B., Frear, D. E. H., Mumma, R. P., Hamilton, R. H., & Cotner, R. C. (1967) *J. Agric. Food Chem.* **15,** 227–230

(10) Wheeler, W. B., Thompson, N. P., Andrade, P., & Krause, R. T. (1978) *J. Agric. Food Chem.* **26,** 1333–1337

(11) Wheeler, W. B., Thompson, N. P., Edelstein, R. L., & Krause, R. T. (1979) *Bull. Environ. Contam. Toxicol.* **23,** 387–390

(12) Wheeler, W. B., Thompson, N. P., Andrade, P., & Krause, R. T. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 1286–1290

(13) Barr, A. J., Goodnight, J. H., Sall, J. P., & Hellwig, J. T. (1976) *User's Guide to the Statistical Analysis System*, Statistical Analysis System Institute, Inc., Raleigh, NC

Gas-Liquid Chromatographic Determination of 4-Chlorophenoxyacetic Acid Residues in Mung Bean Sprouts

YEW-SIN WONG
Ministry of Health, Department of Scientific Services, Outram Rd, Singapore 0316

A gas-liquid chromatographic (GLC) method is described for determining 4-chlorophenoxyacetic acid (CPA) residues in mung bean sprouts. The residues were extracted from samples by using ethyl acetate followed by liquid-liquid partition into 5% $NaHCO_3$ solution. After acidification and reextraction with ethyl acetate, CPA was reacted with pentafluorobenzyl bromide (PFB-Br) to form the PFB derivative. The reaction mixture was separated on a silica gel column to remove excess reagent, and the derivative was eluted with a solution of 75% toluene in hexane. GLC separations were performed on a 3% OV-1 column at 230°C. As low as 10 pg CPA could be quantitated, which is equivalent to about 0.05 ppm at residue level, using an electron capture detector. Recoveries of CPA from fortified mung bean sprouts (0.05, 0.20, and 0.50 ppm) ranged from 71 to 107%. The PFB derivative was further identified by gas chromatography-mass spectrometry.

Mung bean (*Phaseolus* sp.) sprout is a popular fresh vegetable used in oriental cooking. The traditional method of production yields bean sprouts with long and hairy roots, and fastidious consumers would prefer to have the roots picked. Several reports indicate that ethylene or ethefon, an ethylene-generating agent in plant metabolism, and physical pressure could induce morphological responses from hypocotyl and roots of mung bean sprouts (1, 2). Cheng and Chua (3) reported that 4-chlorophenoxyacetic acid (CPA) was most effective in restricting root growth of mung bean sprouts among the 6 plant growth regulators that they screened. These "rootless" bean sprouts had larger diameters and shorter roots.

4-Chlorophenoxyacetic acid is well known as a plant growth regulator and is used mainly for the propagation of cuttings. Little is known about CPA levels in foods. The purpose of this study was to develop a simple, efficient method for determining traces of CPA which are present as residues in rootless bean sprouts.

Methods for cleanup and subsequent gas chromatographic determination of phenoxyacetic acids as their pentafluorobenzyl (PFB) esters have been reported (4–9). The enhanced sensitivity of these PFB derivatives to electron capture detection is particularly suitable for trace analysis.

The present paper describes the cleanup of traces of CPA extracted from mung bean sprouts. The acid was converted to the PFB derivative by reaction with PFB bromide (PFB-Br) in acetone solution in the presence of K_2CO_3. The method allows both electron capture (EC) gas-liquid chromatographic (GLC) quantitation of sub-ppm levels of CPA and confirmation by GLC/mass spectrometry (MS).

METHOD

Reagents

Use redistilled analytical or pesticide grade solvents.

(a) *4-Chlorophenoxyacetic acid standard.*—ICN Nutritional Biochemicals, Cleveland, OH 44128.

(b) *Pentafluorobenzyl bromide.*—Dissolve 1 mL PFB-Br (99+% purity, Aldrich Chemical Co., Milwaukee, WI 53233) in 100 mL acetone. (Caution: Reagent is strong lachrymator.)

(c) *Potassium carbonate solution.*—30%. Dissolve 30 g analytical grade K_2CO_3 in 100 mL water.

(d) *Silica gel.*—70–230 mesh (E. Merck, Darmstadt, West Germany). Activate silica gel 60 by heating overnight (14 h) at 130°C. Let cool in tightly stoppered bottle. Deactivate by adding 5% (w/w) distilled water and mix thoroughly. Equilibrate 24 h before use.

Apparatus

(a) *Mixer blender.*—Waring commercial blender, or equivalent.

(b) *Chromatographic columns.*—Glass tubes, or equivalent, 10 cm × 5 mm id, with piece of glass wool that has been previously washed with acetone and dried.

(c) *Gas chromatograph.*—Perkin-Elmer Model Sigma 3B, equipped with constant current ^{63}Ni electron capture detector and 1.5 m × 3 mm id glass column packed with 3% OV-1 on 80–100 mesh Chromosorb W. Operating conditions: injector 230°C, detector 250°C, column 230°C; nitrogen carrier gas 40 mL/min; connected to

Received January 15, 1982. Accepted March 23, 1982.

0004-5756/82/6505-1118-04$01.00

Spectra-Physics Model SP 4100 computing integrator for data reduction.

(d) *Gas chromatograph–mass spectrometer.*—Hewlett-Packard Model 5985B, operated in electron impact (EI) mode, equipped with 1.5 m × 2 mm id glass column packed with 3% OV-1 on 80–100 mesh Chromosorb W (HP). Helium gas flow 30 mL/min; column 220°C; scanned from m/z 50 to 400.

Preparation of Sample

Weigh and freeze 25 g mung bean sprout samples in 250 mL beaker. Transfer frozen sample to Waring blender and homogenize 5 min with 10 g anhydrous Na_2SO_4 and 100 mL ethyl acetate. Decant extract into 500 mL separatory funnel; add 100 mL ethyl acetate to residue and repeat extraction. Wash combined ethyl acetate extracts with 50 mL water. Extract CPA from washed ethyl acetate fraction with two 50 mL portions of 5% $NaHCO_3$ solution. Reject ethyl acetate fraction. Adjust aqueous fraction to ca pH 3 with 20 mL 30% H_3PO_4, add 5 g Na_2SO_4, and re-extract with two 25 mL portions of ethyl acetate. Wash combined ethyl acetate extracts with 5 mL water and dry over anhydrous Na_2SO_4.

Derivatization

Pipet 2 mL ethyl acetate extract (equivalent to 1 g sample) into 50 mL centrifuge tube and evaporate to dryness at 30°C with gentle stream of dry nitrogen. Dissolve residue in 2 mL acetone, and add 50 μL PFB-Br solution and 30 μL 30% K_2CO_3 solution. Stopper tube and shake to mix. Let contents react at room temperature for 3 h or overnight, whichever is more convenient. Then add 2 mL isooctane, and evaporate to ca 1 mL at 30°C under gentle stream of dry nitrogen. Add another 2 mL isooctane and repeat evaporation to final volume of ca 1 mL.

Column Cleanup and Determination

Place glass wool plug in chromatographic column and add 5 cm deactivated silica gel. Tap to settle solid. Wash column with 5 mL hexane; transfer isooctane extract from derivatization onto column by using clean disposable pipet. Wash tube with two 1 mL portions of hexane and add washes to column. Elute column with 15 mL toluene–hexane (1 + 3) to remove excess reagent and discard eluate (Fraction I). Then elute with 8 mL toluene–hexane (3 + 1); collect eluate (Fraction II) in graduated tube, adjust volume to 8 mL, and analyze directly by injecting 2 μL of Fraction II into gas chromatograph. Compare retention times and peak areas and/or peak heights against external standards (0.05–0.50 μg CPA/2 mL ethyl acetate) processed through derivatization and column cleanup steps.

After determination, confirm presence of PFB derivative of CPA by evaporating Fraction II at 40°C under dry nitrogen to near dryness; dissolve residue in 20–50 μL hexane and inject 5 μL of extract into GC/MS system.

Results and Discussion

In the method described, cleanup of the crude extract by liquid–liquid partitioning satisfactorily eliminated background interferences. Direct extraction of mung bean sprout samples with acetone yielded co-extractives which overwhelmed subsequent attempts at chromatography.

The high polarity or low volatility of the phenoxyacetic acids prevents direct GLC determination. The formation of the more volatile trimethylsilyl and methyl ester derivatives from extracts were not sufficiently sensitive for residue level determination largely because of the unresolved co-extracted peaks. Chau and Terry (5) reported the formation of pentafluorobenzyl ester derivatives of phenoxyacetic acids by reaction with PFB-Br in acetone in the presence of aqueous K_2CO_3. We found the enhanced sensitivity of the PFB derivative of CPA to electron capture detection suitable for trace analysis. A linear response, as peak area and/or peak height, was obtained over the range 0–0.70 ppm (r = 0.997). The minimum quantitative detection was about 10 pg which is equivalent to about 0.05 ppm at residue level for a 25 g sample. Other than the 30 μL of aqueous K_2CO_3 reagent used in the reaction, moisture affected quantitative derivatization. Residual moisture in the final ethyl acetate extracts was removed by drying over anhydrous Na_2SO_4.

A column cleanup was also used in this study (10). The unreacted and strong electron-capturing PFB-Br reagent was removed by using a 5 cm × 5 mm id minicolumn of deactivated silica gel and 15 mL toluene–hexane (1 + 3). An equivalent of 0.20 ppm CPA, i.e., 0.20 μg CPA, as PFB derivative, was loaded onto the silica gel column and the following elution data (%) were obtained: 0–1 (mL fraction), 9; 1–2, 45; 2–3, 27; 3–4, 10; 4–5, 6; 5–6, 2; 6–7, 1; 7–8, none detected. Percent recovery averaged 87 ± 5 (mean ± SD, n = 6). The relatively polar acetone solvent in the extracts after derivatization must be replaced with isooctane before column chromatography.

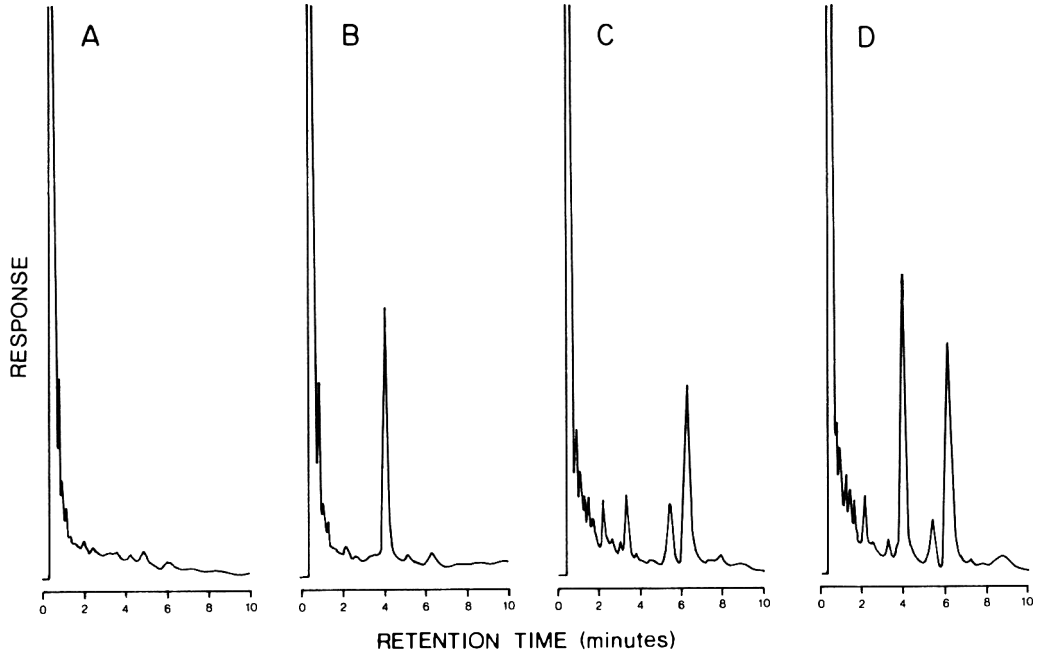


Figure 1. EC chromatograms obtained by described method (3% OV-1 at 230°C): A, reagent blank; B, CPA standard, retention time 4.03 min; C, unfortified sample of mung bean sprouts; D, sample fortified with 0.20 ppm CPA; similar chromatogram as D for "rootless" sample.

We noted that silicone liquid phases of intermediate polarity (OV-17) did not adequately resolve PFB derivative of CPA from other chromatographic peaks; false positives were observed on numerous occasions. The low-polarity silicone phase of OV-1 performed better, as shown in Figure 1, where CPA (retention time, 4.03 min) is well resolved from background contribution. The numerous peaks, which were not removed by the column cleanup and were still present as background in the extracts of bean sprouts, are probably PFB esters of other acidic constituents that were co-extracted. An examination of the single ion mass fragmentation at m/z 181 also indicated the possible presence of various PFB esters formed with acidic components other than the PFB derivative of CPA (retention time, 4.19 min).

Application of the derivatization procedure to the determination of CPA in fortified mung bean sprouts gave good results. As little as 0.05 ppm could be detected; 0.05, 0.20, and 0.50 ppm CPA added to bean sprouts gave recoveries of 71–94% (mean 84 ± 8, $n = 6$), 85–107% (mean 97 ± 8), and 92 – 102% (mean 97 ± 4), respectively. The effects of CPA on the growth of hypocotyl and roots of mung bean sprouts have been reported (3). Various samples of mung bean sprouts made rootless by treatment with CPA were examined for CPA residues by using the proposed method. Twenty treated samples were analyzed and contained between 0.05 and 0.48 ppm CPA. CPA residues between 0.21 and 0.39 ppm in 7 samples were identified by GLC/MS.

The structure of the PFB derivative of 4-chlorophenoxyacetic acid ($ClC_6H_5OCH_2COOCH_2C_6F_5$) was confirmed by mass spectrometry: the molecular ion occurs at m/z 366 and the fragments at m/z 181 ($—CH_2C_6F_5$), 141 ($—COOCH_2C_6F_5$), and 111 ($—OCH_2COOCH_2C_6F_5$). The distinctive mass spectrum of the eluted peak at retention time 4.19 min in extracts of rootless mung bean sprouts was identical to that of the PFB derivative of CPA standard.

In conclusion, the presence of CPA residues in rootless mung bean sprouts can be determined by forming the PFB-Br derivative, which showed high sensitivity to EC detection. The residue was confirmed by GLC/MS.

Acknowledgments

The author thanks M. C. Dutt, Director, Department of Scientific Services, for his keen interest and encouragement; and Y. W. Cheng, Sembawang Field Experimental Station, Agriculture Division, Primary Production Depart-

ment, for the samples of "rootless" mung bean sprouts. The author also thanks Peter Lai for his help in the analysis of control samples, and K. L. Tham for preparing the figure.

REFERENCES

(1) Chang, C. N., & Lin, R. S. (1977) *J. Chin. Soc. Hort. Sci.* **23,** 19–143
(2) Helms, K., & Meyers, L. F. (1973) *Thai J. Agric. Sci.* **6,** 293–302
(3) Cheng, Y. W., & Chua, S. E. (1980) *Singapore J. Primary Ind.* **8,** 48–56
(4) Kawahara, F. K. (1968) *Anal. Chem.* **40,** 1009–1010
(5) Chau, A. S. Y., & Terry, K. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 633–636
(6) Coburn, J. A., Ripley, B. D., & Chau, A. S. Y. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 188–196
(7) Agemian, H. & Chau, A. S. Y. (1976) *Analyst (London)* **101,** 732–737
(8) Cotterill, E. G. (1979) *J. Chromatogr.* **171,** 478–481
(9) Mohammad, A. S., & Paasivirta, J. (1979) *Anal. Chem.* **51,** 598–602
(10) Johnson, L. G. (1973) *J. Assoc. Off. Anal. Chem.* **56,** 1503–1505

Extraction Procedures for Oilseeds and Related High Fat-Low Moisture Products

LEON D. SAWYER
Food and Drug Administration, 240 Hennepin Ave, Minneapolis, MN 55401

A combined sample preparation/extraction procedure is presented for pesticide residue analysis of oilseeds and related high fat-low moisture products. The procedure utilizes high-speed milling to prepare the sample and high-speed homogenization in the extraction step to achieve what is apparently quantitative isolation of both incurred residues and natural oils. A separate, simple, oil determination step allows findings to be reported on either the fat or whole product basis. Petroleum ether, ethyl ether-petroleum ether (1 + 1), and ethanol are used serially as the extractants. Usual fatty food cleanup procedures and multiresidue gas chromatographic detection techniques are utilized. The procedure presented in this paper is a refinement of earlier work which used a homogenizer both to grind and to extract samples of unground seeds and which demonstrated essentially complete extraction of endrin residues in soybeans and DDT residues in mustard seed. Identical samples analyzed by the currently recommended shakeout procedure, 29.012, gave recoveries of approximately 50% of the total residues. The procedure presented in this paper was satisfactorily tested on 13 different oilseed types and one sample of soda crackers. Oil content for these samples ranged from 5 to 69%.

Methods for oilseeds, nuts, and high-fat feeding materials referenced in the *Pesticide Analytical Manual* (PAM) (1) acknowledge that the optimum extraction procedure for pesticide residues in these product types has not been established and recommend that the method developed for cheese be utilized for these product types. This recommended method is identical to that appearing in *Official Methods for Analysis* (2) in **29.012.** The method involves a triple extraction of a ground sample by shaking with mixed ethers in the presence of ethanol to isolate the oil or fat. Analysis then proceeds as with a usual dairy fat sample except that a calculation to the whole product basis is necessary for reporting results. This procedure is based on the assumptions that any pesticide residues that are present will be uniformly extracted with the oil, and that the oil content of the sample will be known. In addition, when this procedure was recommended, there was no effective or efficient means available to uniformly grind or comminute samples such as oilseeds to the recommended 20 mesh particle size. The following report is intended to demonstrate that the above assumptions are not accurate with all sample types for which the method was recommended and to propose a sample preparation/extraction procedure for these products that can be readily incorporated into existing PAM (1) and AOAC (2) cleanup and determinative procedures.

Early investigation into the recommended method with soybeans containing endrin and with mustard seed containing DDT isomers showed that only about 50% of these field-incurred pesticides were recovered. Further investigations revealed that the poor extractability was primarily related to inadequate interaction between the sample particles and the solvents. By defining a sample preparation procedure, changing the order and amounts of extraction solvents, and substituting rigorous homogenization for the blending and shaking steps, the cheese procedure was modified to one that apparently gives quantitative recoveries of both natural oils (lipids) and incurred residues, i.e., pesticides of the nonpolar organochlorine class and certain organophosphorus pesticides.

In the early stages of the investigation, a closed extraction vessel with a Sorvall/Omni-mixer served as both a grinder and a blender for samples of unground seeds. When a mill became commercially available that could effectively grind difficult samples like the oilseeds, a Polytron homogenizer was substituted, which uses an open extraction vessel and eliminates pressure buildup due to solvent evaporation that occurs with a closed system. Both techniques appear to be equivalent for extracting residual pesticides, but the latter approach gives greater assurance of homogeneity with larger laboratory samples and is not as demanding on the blender.

Received August 31, 1981. Accepted March 30, 1982.
This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.
The recommendation of the Associate Referee was approved by the General Referee and Committee E and was accepted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) **65**, 383.

METHOD

Reagents and Apparatus

(a) *Solvents.*—High purity, glass-distilled petroleum ether, ethyl ether, and acetonitrile.

0004-5756/82/6505-1122-07$01.00

Reagent grade ethanol (USP 95%) is satisfactory. Criteria for purity are described in PAM I, sec. 120.

(b) *Grinder.*—Mill capable of grinding samples in a homogeneous fashion to 1 mm particle size without excessive heat buildup or oil separation. Ultra Centrifugal Mill (distributed by Quartz Technology, Inc., 100 Grand St, Westburg, NY 11590), or equivalent.

(c) *Homogenizer.*—Either Polytron Model PT 10-35 (distributed by Brinkmann Instruments, Inc., Westburg, NY 11590) or Sorvall/Omni type (DuPont Instruments-Sorvall Biomedical Div., Newton, CT 06470). Either PT 35 or PT 20 ST homogenizer heads may be used with the Polytron instrument. The heads should be equipped with a metal bushing because Teflon bushings may wear excessively and cause the shaft to bind.

(d) *Extraction vessel.*—Stainless steel 400 mL Sorvall/Omni chamber for use with either homogenizer. A 500 mL glass centrifuge bottle can be used with the Polytron if a PT 20 ST homogenizer head is used.

Sample Preparation

Mix sample well and grind in mill (b) equipped with 1 mm sieve ring. If noticeable heat builds up, alternatively grind without sieve ring, or use larger ring (such as 3 mm) and then regrind through 1 or 0.5 mm sieve ring. Maintain rotor speed of mill at 20 000 rpm to aid in cooling.

Extraction

Weigh 50 g ground sample in extraction vessel (d). Add 200 mL petroleum ether and blend 1.5 min at high speed. Centrifuge extraction vessel and decant solvent into 500 mL beaker. Add 150 mL ethyl ether–petroleum ether (1 + 1) to vessel and blend, centrifuge, and decant into beaker as previously described. Set beaker containing combined ethers under gentle air stream to concentrate to total solvent volume of ca 100 mL. Re-extract residue in vessel with 150 mL ethanol for 1.5 min, centrifuge, and decant into a 1 L glass-stopper separatory funnel. Add 50 mL ethanol to vessel. Wash residue in vessel by gently blending. Then centrifuge and decant into same separatory funnel.

Add concentrated ethers from first 2 extractions to separatory funnel, using small (ca 5 mL) petroleum ether washes of beaker. Mix well and add 600 mL water and ca 40 mL saturated NaCl solution. Hold 1 L separatory funnel in horizontal position and mix thoroughly 45 s. Let layers separate and drain aqueous portion into second 1 L separatory funnel containing 100 mL petroleum ether. Mix thoroughly ca 15 s and let layers separate. Drain and discard aqueous portion and drain petroleum ether into original separatory funnel. Wash combined ethers with two 100 mL portions of water. Dry ethers by passing solution through column of anhydrous Na_2SO_4, and concentrate ethers, using one of the following alternatives: (*1*) Remove solvent completely and consider residue remaining to represent 50 g sample; or (*2*) concentrate solvent to total volume of ca 25–30 mL, transfer to graduated cylinder by using small petroleum ether washes, and dilute to known volume.

If alternative (*2*) is used, an aliquot can be easily transferred to a tared beaker for oil content determination and another aliquot can be used for further cleanup. Aliquots taken represent a direct ratio of the original 50 g sample, e.g., 50 g to 100 mL = 0.5 g/mL.

Continue with analysis as described in **29.014,** petroleum ether–acetonitrile partitioning using ≤3 g total oil. The aliquot alternative allows a simple 1 step determination for calculation of the oil content of samples for which this value is unknown. The calculated oil content can then be used to determine the proper aliquot needed for the partitioning step. This approach also allows results to be based on either the fat (oil) or whole product basis. (Note: Other cleanup procedures may be just as applicable as the petroleum ether–acetonitrile partitioning. Preliminary work has shown gel permeation chromatography to be effective for dieldrin residues in soybeans.)

Results and Discussion

Rogers (3) investigated the extraction of field-incurred aldrin from canary seed and related its extractability to proper mesh size of the ground sample. He reported that if the material was not finely particulated, extraction efficiency was reduced. However, the hard outer shell could not be ground to 30 mesh and was consequently separated from the ground pulp containing the aldrin. Thus this separation produced a disproportionate distribution of the aldrin in the sample and thereby prevented accurate calculations based on whole product weight. Problems of representative grinding are magnified with samples of oilseeds because heat buildup and oil separation readily occur when attempts are made to grind them by conventional means.

Smith (4) addressed the problems associated

Table 1. Comparative particle size reduction and percent oil determined by Wiley/29.012 method [a] vs Omni/petroleum ether procedure [b]

Oilseed sample	Wiley/29.012		Omni/petroleum ether	
	Dried pulpy residue, [c] %	Oil, %	Dried pulpy residue, [c] %	Oil, %
Mustard seed	31	21.6	57	28.0
Mustard seed	41	16.1	56	26.9
Cocoa beans	23	25.1	63	48.4
Soybeans	19	11.5	82	17.8
Soybeans	28	11.6	77	18.5

[a] 50 g sample. A Wiley mill was used for grinding, and AOAC **29.012** was used for extraction.
[b] 50 g sample. A Sorvall/Omni homogenizer was used for cracking and grinding the unground seeds and for extracting (3×) with petroleum ether.
[c] <40 mesh. (The remaining pulpy residue was >40 mesh.)

with grinding cocoa beans (about 50% oil) and reported that the addition of dry ice in the grinding step minimized the formation of heat and problems of oil separation. However, because of the expense and limited availability of dry ice, it was recognized that this procedure should not be considered routine. Further work in our laboratory with mustard seed (about 30% oil) indicated that, even with dry ice, satisfactory grinding could not be easily achieved. Smith (4) used both a Straub mill and a Wiley mill; our experience with mustard seed was obtained with a Wiley mill and a Burr mill.

Because a routine grinding procedure was not readily available, the possibility of using the extraction step itself was investigated as a means of particle size reduction. In the investigation, a Sorvall/Omni homogenizer served simultaneously as a grinder and an extraction vessel. The design of the homogenizer with its sealed blending chamber allowed cracking and grinding of the seeds before solvent addition. Subsequent petroleum ether extractions (3×), followed by centrifuging and decanting, showed that this technique effectively reduced the oilseed particle size and increased the efficiency of oil isolation, compared with that obtainable in the AOAC method with preground seeds. Table 1 lists the comparative data obtained with 5 samples of oilseeds: 2 mustard seed samples, 2 soybean samples, and 1 cocoa bean sample. With the cocoa bean sample, almost twice as much oil and almost 3 times the amount of fine (<40 mesh) material were isolated after the extraction with the Omni/petroleum ether procedure. (The remaining pulpy material was >40 mesh.) The mustard seed and soybean samples also demonstrate a similar difference in results. The oil content values obtained by the Omni/petroleum ether procedure for soybeans, 17.8 and 18.5%, compare favorably with that reported by Morrison (5), 18.0%, and that appearing in PAM I (1), 17.7%.

Because these data suggested that complete oil removal could be obtained by the use of the Omni/petroleum ether procedure, we assumed that complete pesticide extraction would also be accomplished. To verify or contradict this assumption, we extracted one sample of mustard seed containing incurred DDT residues and one sample of soybeans containing incurred endrin residues by both the Omni/petroleum ether and AOAC procedures. In addition, the solid residues remaining after the triple extractions were air-dried and then exhaustively extracted with methanol–chloroform as described in PAM I, sec. 253. Identical cleanup of all 4 extracted portions was accomplished by using techniques described in **29.014,** and the recovered pesticide residues in each portion were quantitated by electron capture gas chromatography as described in **29.018.** Each result was calculated as parts per million on the basis of the original sample weight, i.e., the solvent portions and the dried pulpy residue portions were each considered equivalent to 50 g. Table 2 lists the results obtained and shows that only 90% of the total *p,p'*-DDT and 80% of the total endrin were recovered in the oil portions by using the Omni/petroleum ether procedure. With the AOAC procedure, the pesticide residues in both samples were split almost evenly between the oil and the pulp portions. The 80% recovery value for endrin in soybeans indicates that, even with complete oil extraction from the seeds, significant amounts of translocated residues can be closely bound to the dry pulpy part of the seeds. Consequently, alternative solvent systems of greater polarity were investigated by testing endrin-containing soybeans.

Table 2. Comparative recoveries of incurred pesticide residues by Omni/petroleum ether and Wiley/29.012 methods [a]

Method	DDT in mustard seed, ppm [b]	Endrin in soybeans, ppm [b]
Omni/petroleum ether	0.094	0.099
Soxhlet of pulp [c]	0.012	0.026
Total	0.106	0.125
Wiley/**29.012**	0.051	0.067
Soxhlet extn of pulp [c]	0.054	0.065
Total	0.105	0.132

[a] See Table 1, footnotes *a* and *b*.
[b] Whole product basis, i.e., relative to 50 g sample originally weighed.
[c] Pulpy material was exhaustively extracted as described in PAM I, sec. 253.

Solvents that were investigated and the results obtained are shown in Table 3. Trial 1 is a repeat of the procedure discussed in relation to Table 2 data. The trial 1 result was obtained with a different sample of soybeans from that used for trials 2–5; therefore, the total endrin amounts recovered should not be compared. Each extraction trial followed the format previously discussed: Extract with solvents, dry, and exhaustively extract pulpy material; clean up both portions by AOAC methods, and quantitate endrin by using 50 g sample weights. All blending times were 3 min, except trial 4, in which a 1 min blend with water was followed by a 3 min blend with acetonitrile.

Trials 1 and 2, corresponding to triple extractions with petroleum ether and with ethyl ether–petroleum ether (1 + 1), obtained essentially the same amounts of extractable endrin in the solvent, 79.2 and 76.7%, respectively. In addition, in trial 2, in which a known amount of *p,p'*-DDE (0.04 ppm) had been added, a 105% recovery was obtained from the solvent portion. In trial 3, 2 petroleum ether extractions were followed by the addition of water and acetonitrile. Only slightly better recovery, 81.2%, of the endrin was solvent-recovered. Trial 4 eliminated the water used in trial 3, and improved duplicate recoveries of 89.4 and 85.9% were obtained. Trial 5 used a "bridged" solvent system of petroleum ether, ethyl ether–petroleum ether (1 + 1), and ethanol. This system demonstrated triplicate recoveries of 94.2, 91.4, and 95.3% of the endrin in the solvent portion. A repeat of trial 5 with mustard seed containing DDT (*o,p'*- and *p,p'*-isomers) showed triplicate total extraction (100% of the DDT) in the solvent portion.

The data obtained from trial 5 (and related studies) showed that the solvent system both removes the oil and effectively wets the pulverized pulpy seed material to provide a nearly 100% extraction. The Sorvall/Omni homogenizer, however, did not provide the durability needed for the continual rigorous grinding duties that are required by this approach. Consequently, an alternative approach which readdressed the problem of prior grinding was initiated.

Investigation into the commercially available laboratory mills showed that an Ultra Centrifugal Mill or Micro-Jet 10 was capable of efficiently grinding these sample types without the heat buildup or oil separation problems that were encountered earlier. Preliminary investigation into this mill was reported previously (6). However, again by using soybeans containing

Table 3. Solvent system trials with incurred endrin in soybeans, using unground beans and Sorvall/Omni homogenizer

Trial [a]	Solvent system	Volumes	Endrin found, ppm [b] Omni/solv. portion	Soxhlet/pulp portion [c]	Total	% of total in solv. portion
1	petroleum ether (3×)	200, 100, 100	0.099	0.026	0.125	79.2
2	petroleum ether-ethyl ether (1 + 1)(3 ×)	200, 100, 100	0.138	0.042	0.180	76.7 [d]
3	petroleum ether (2×), water, acetonitrile	200, 100, 50, 150	0.138	0.032	0.170	81.2
4	petroleum ether (2×), acetonitrile	200, 100, 200	0.152	0.018	0.170	89.4
	Duplicate		0.146	0.024	0.170	85.9
5	petroleum ether, ethyl ether-petroleum ether (1 + 1), ethanol	200, 100, 200	0.161	0.010	0.171	94.2
	Duplicate		0.159	0.015	0.174	91.4
	Triplicate		0.162	0.008	0.170	95.3

[a] Trial 1 from Table 2. Trials 2–5 were conducted with a different lot of soybeans.
[b] Whole product basis, i.e., relative to 50 g sample originally weighed.
[c] Pulpy material was exhaustively extracted in Soxhlet as described in PAM I, sec. 253.
[d] Recovery of 0.04 ppm spike of *p,p'*-DDE was 105% (0.042 ppm), all in the solvent portion.

Table 4. Recoveries of added pesticides, using preground [a] samples and the described method, and one comparative recovery by the 29.012 method

Product	Pesticide	Added, ppm	Rec., ppm	Rec., %	Oil detd, %
Soybeans	endosulfan I	0.107	0.098	91.6	—
Crackers	*trans*-chlordane	0.095	0.083	87.4	—
Soybeans	diazinon	0.018	0.020	111.0	—
Soybeans	methoxychlor	0.393	0.375	95.4	14.0
Soybeans [b]	methoxychlor	0.785	0.536	68.3	7.0

[a] All samples were ground with the Ultra Centrifugal Mill.
[b] Recovery by the **29.012** method.

endrin, it was found that, even with a finely ground sample (99.3% was less than 40 mesh and 41.8% was less than 100 mesh), duplicate recoveries of only 86.4 and 82.7% of the total endrin were obtained with solvent extraction by the mixed solvent system described in trial 5 of Table 3. In this case, the lower recoveries were attributed to the use of a laboratory Waring blender, which did not appear to provide the vigorous mixing action that was experienced with the Sorvall/Omni homogenizer. Concurrent duplicate *p,p'*-DDE fortifications (0.782 ppm) recovered in the solvent portion were 94.3 and 99.1%.

Substitution of a Polytron homogenizer for the Waring blender provided the necessary mixing action and its use is currently recommended. Any glass or stainless steel vessel that is compatible with the homogenizer head and with the centrifuge used can serve as the extraction vessel. Both 500 mL centrifuge bottles and 400 mL Sorvall cups have been used successfully. Additional refinements to the original procedure were made to accommodate the use of the Polytron homogenizer: Blending times were reduced to 1.5 min and the volume of the ethyl ether–petroleum ether (1 + 1) (second extraction) was increased to 150 mL. These refinements reduced a binding problem with the shaft of the Polytron homogenizer without any apparent loss of extraction efficiency.

Some of the various sample types examined successfully by using this method (with the Polytron homogenizer) are soybeans, flax seed, rapeseed, mustard seed, canary seed, sunflower seed, walnut meats, sesame seed, caraway seed, peanuts, crackers, and cocoa beans. Although the results of all the oil content determinations were not recorded, the oil content of those samples listed above ranged from 5% for the canary seed to 69% for the walnut meats. Some of the residues detected in these samples were isomers of BHC, malathion, parathion, toxaphene, DDTs, aldrin, dieldrin, endrin, heptachlor, heptachlor epoxide, methoxychlor, endosulfans, parathion, methyl parathion, PCNB, and chlordane-related compounds.

Table 4 lists typical recoveries obtained by the method described here and a single comparative recovery obtained by the AOAC method. Recoveries of the 4 pesticides endosulfan I, *trans*-chlordane, diazinon, and methoxychlor ranged from 87.4% for *trans*-chlordane to 111.0% for diazinon. The one comparative recovery from soybeans fortified with methoxychlor showed that the AOAC method with its shakeout extraction recovered only 68.3% of the added amount vs 95.4% for the Polytron homogenizer extraction. The AOAC method also recovered only 50% of the oil content when compared with the more vigorous Polytron homogenizer approach.

Table 5 demonstrates the reproducibility of the method for 8 samples of sunflower seeds containing malathion. The duplicate (different analysts) results for the 8 samples show that the most divergent pair of results was for Sample 4, for which the original result was 22% higher than the duplicate.

Table 5. Duplicate [a] malathion findings for 8 samples of sunflower seeds by the described method

Sample	Malathion, ppm		% Agreement [b]
	Original	Duplicate	
1	0.72	0.74	97
2	1.14	1.11	103
3	0.83	0.80	104
4	1.10	0.90	122
5	0.98	0.83	118
6	0.71	0.62	115
7	7.9	6.9	114
8	9.3	8.3	112

[a] Different analysts.
[b] % agreement = (original/duplicate) × 100.

Table 6. Comparative pesticide and oil findings for sunflower seeds, [a] obtained by extraction procedure 29.012, [b] by the described method with a Polytron homogenizer, [c] and by the described method with a Waring blender [d]

				Described method			
		29.012		With Polytron homogenizer		With Waring blender	
Sample	Pesticide	Pesticide, ppm	% Oil	Pesticide, ppm	% Oil	Pesticide, ppm	% Oil
1	methoxychlor	0.16	37.1	0.19	41.8	—	—
2	malathion	0.23	36.7	0.24	39.2	—	—
3	malathion	8.25	35.0	9.44	40.2	7.53	38.4

[a] All analytical portions were ground with the Ultra Centrifugal Mill through a 1 mm sieve.
[b] AOAC shakeout **29.012.**
[c] The Polytron homogenizer was used in the extraction step.
[d] Used in place of the Polytron homogenizer.

Again for samples of sunflower seeds, Table 6 shows additional method comparability findings. In this table, 3 samples (2 containing malathion and one containing methoxychlor) were compared for total parts per million found and for oil content by using the shakeout procedure and the method described here. One of the samples containing malathion residues was also analyzed by the method described here, except that a laboratory Waring blender was used instead of the Polytron homogenizer. All analytical portions were ground with the Ultra Centrifugal Mill through a 1 mm sieve. In these cases, better agreement for the oil content was obtained than was previously experienced with other seeds, but the Polytron homogenizer values are higher in all cases. If the results obtained for the AOAC method are calculated on the oil basis and converted to the whole product basis by using the oil percentages derived from the Polytron homogenizer approach, good analytical agreement is achieved. However, this indicates that these residues are totally oil-solubilized or that they are surface contaminants and is contrary to the findings for endrin in soybeans previously discussed.

A final extraction/applicability test with the method described here was conducted with 2 samples of peanuts. The samples were extracted as described under *Method* and the remaining pulp was then exhaustively extracted with chloroform–methanol (1 + 1) as described earlier. The results from this test appear in Table 7. The method was shown to extract 96.5, 97.1, and 98.5%, respectively of α-BHC, β-BHC, and dieldrin residues that were known contaminants in the 2 samples. Because of the low residue levels (<0.1 ppm), the total micrograms extracted from each portion are compared. The values obtained from the Soxhlet extraction are calculated from chromatographic peaks that were present at the same absolute retention time as the standard materials and could have been attributable to background.

Conclusions

Any method that bases its residue findings on the amount of oil analyzed and converts back to

Table 7. Recoveries of incurred pesticide residues in peanuts by the described method with Soxhlet extraction [a] of pulpy material

			Pesticide residue extracted, μg		
Sample	Pesticide residue	% Oil	Polytron/ solv.	Soxhlet/ pulp [b]	% of total in solv. portion
1	α-BHC	41.3	2.05	0.0738	96.5
	β-BHC		3.95	0.1197	97.1
2	dieldrin	40.1	3.22	0.0483	98.5

[a] Pulpy material was exhaustively extracted in Soxhlet as described in PAM I, sec. 253.
[b] Results reported are "apparent" residues because gas chromatographic peaks appeared at the retention time of the standard and were calculated as such.

the whole product basis from a published value may be based on some erroneous assumptions. For instance, laboratory findings usually show soybeans to contain 17–18% oil when marketed. However, the oil content of soybeans depends on the amount of dehydration that occurs. Thus the oil content of soybeans has been found to be as high as 24%, and, with greener beans, has been as low as 14%. Sunflower seeds are another crop in which oil content levels vary. This crop is marketed in several forms (7): wet out of the field, dried-in-hull, as low-oil confectionary types, as high-oil types to be further processed, and in various combinations. Depending on the particular instance, oil content can range from about 25 to 52%.

The method described here should be uniformly adaptable to all types of oilseed (and related) products. All indications are that essentially 100% of the residues are extracted, regardless of the oil content, which need not be known but can easily be determined.

Interlaboratory experiences with the method that were relayed to the Associate Referee from several Food and Drug Administration laboratories indicated that very few problems have been encountered with its usage. Some emulsion problems in the ethanol flood-out step were reported with certain peanut samples. The most frequent complaint has been in regard to the time needed for analysis, which is acknowledged to be somewhat longer than that for most extraction procedures, but is no longer than the currently recommended procedure. On the basis of the improved extractability demonstrated by the proposed procedure, the Associate Referee recommends that the method be collaboratively studied within the next year to expand **29.012** to include oilseeds and some related high fat–low moisture products.

Acknowledgments

The author wishes to acknowledge the work of present and past members of the Pesticide Laboratory, Minneapolis District, FDA, who generated much of the data presented and offered many helpful suggestions during the development of this procedure. Also acknowledged are M. Hennessey, Baltimore District, FDA, and J. Gaul, New Orleans District, FDA, for supplying samples of peanuts and soybeans.

References

(1) *Pesticide Analytical Manual* (Rev. 6/80) Vol. I, Chapter 2, Food and Drug Administration, Washington, DC

(2) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, Chapter 29

(3) Rogers, W. M. (1969) Laboratory Information Bulletin 836, Food and Drug Administration, Washington, DC

(4) Smith, R. E. (1976) Laboratory Information Bulletin 1902, Food and Drug Administration, Washington, DC

(5) Morrison, F. B. (1959) *Feeds and Feeding*, 22nd Ed., Morrison Publishing Co., Clinton, IA, p. 1064

(6) Sawyer, L. D. (1977) Laboratory Information Bulletin 2023, Food and Drug Administration, Washington, DC

(7) Extension Bulletin 25 (Rev. 7/78) D. W. Cobia (Ed.), North Dakota State University, Fargo, ND

MICROBIOLOGICAL METHODS

Use of Iron Milk Medium for Enumeration of *Clostridium perfringens*

WILLIAM D. ST. JOHN, JACK R. MATCHES, and MARLEEN M. WEKELL[1]
University of Washington, Institute for Food Science and Technology, College for Ocean and Fishery Sciences, Seattle, WA 98195

A simple iron milk medium was used for isolation and enumeration of *Clostridium perfringens* from soil, sludge, and water samples. The whole milk contained only iron powder as a reducing agent; no other inhibitors were added. The iron milk most probable number (MPN) procedure was compared with 4 plating media: sulfite-polymyxin-sulfadiazine, Shahidi-Ferguson perfringens, tryptose-sulfite-cycloserine (both with and without egg yolk), and tryptone-sulfite-neomycin. The selectivity of the iron milk relies solely on the rapid growth of *C. perfringens* at 45°C and the stormy fermentation reaction within 18 h. Isolates were confirmed as *C. perfringens* by standard biochemical tests. The iron milk MPN procedure compared very well with the 4 plating media tested. Selectivity of incubation temperature, short incubation time, and ease of identification by the characteristic stormy fermentation make this method ideal for enumerating *C. perfringens* from large numbers of samples.

During early studies with *Clostridium perfringens*, a need for a rapid, simple, and accurate assay became evident. The selective media devised by several authors for enumerating *C. perfringens* use sulfite and iron. Sulfite, when reduced to sulfide by *C. perfringens*, combines with iron to form the black color surrounding the colonies. In addition, antibiotics are added to inhibit competing facultative organisms.

Several media used by other workers were employed in our studies: sulfite-polymyxin-sulfadiazine (SPS) (1); Shahidi-Ferguson perfringens (SFP) (2), tryptose-sulfite-cycloserine (TSC) with or without egg yolk to indicate lecithinase (3), and tryptone-sulfite-neomycin (TSN) (4). Each of these media also requires confirmatory biochemical tests, which makes processing large numbers of samples time-consuming.

To circumvent any problem associated with chemical inhibitors, a method was developed based on one selective condition, temperature. The optimum temperature for the growth of all strains of *C. perfringens* studied is 45°C (5). At this temperature, the "phoenix" phenomenon (6) occurs, with an initial decline in numbers to minimum counts at 4 h followed by a sudden increase in growth to maximum counts at 6 h.

A unique reaction, stormy fermentation in milk, is used to indicate the presence of *C. perfringens*. This reaction involves the formation of acid, resulting in a curd which is violently broken apart by gas produced by *C. perfringens* and several other clostridia. These 2 conditions, high optimal incubation temperature and stormy fermentation, both unique to *C. perfringens*, virtually eliminate all competitive organisms and the need for inhibitors and confirmatory testing.

In this study we quantitated *C. perfringens* with the iron milk medium, using a 3-tube MPN (most probable number) technique. Data were compared with those collected using 4 other methods. Selected biochemical tests were used to confirm bacterial isolates as *C. perfringens*.

Experimental

Materials

(a) *Milk medium.*—Pasteurized homogenized whole milk was dispensed in 10 mL portions into 16 × 150 mm screw-cap test tubes. Elemental iron powder was added as a reducing agent. Studies showed that ca 0.2 g per tube gave the best results. It has been reported that the amount of iron can affect the carbohydrate metabolism and the levels of gas evolved (7). The medium was sterilized 10 min at 116°C and was stored at room temperature until used. The medium was then steamed for 5 min and held at 45°C ≤ 1 h before use. A 3-tube MPN method was used. Samples were incubated 16–18 h in a 45°C water bath. An alternative procedure (8) is to dispense the milk into sterile tubes, add iron powder, steam 5 min, and hold at 45°C ≤ 1 h before use. Recovery of *C. perfringens* was better when the medium was heated only once.

C. perfringens produces acid in the milk and forms a curd. The resulting decrease in pH to approximately 5.0 becomes inhibitory to *C. per-*

[1] School of Nutritional Sciences

Received October 27, 1981 Accepted February 22, 1982.

0004-5756/82/6505-1129-05$01.00

Table 1. Comparison of *C. perfringens* counts obtained with the high temperature milk MPN technique and with 4 plating media [a]

Sample No.	Sample	Log of counts confirmed as *C. perfringens*[b]				
		A	B	C	D	E
1	Sludge	5.38	5.32	5.33	5.30	5.3
2	Soil	6.0	6.13	6.18	6.08	6.10
3	Soil	6.2	6.23	6.15	6.11	6.08
4	Soil	6.49	6.54	6.48	6.41	6.45
5	Water	5.80	5.71	5.85	5.78	5.73

[a] A = milk MPN method; B = tryptone–sulfite–cycloserine (TSC) with egg yolk; C = tryptone–sulfite–cycloserine (TSC) without egg yolk; D = sulfite–polymyxin–sulfadiazine (SPS); E = tryptone–sulfite–neomycin (TSN).
[b] Values represent the average of duplicate samples.

fringens on standing. Therefore, samples to be stored or tested were transferred to appropriate media within 24 h.

(b) *Blood agar medium.*—Blood agar plates were prepared with blood agar base (BBL) prepared with 5% outdated whole citrated human blood. Sterile glass rods were used to spread 0.1 mL portions of the appropriate dilutions on the surface of the blood agar. Prepared plates were incubated in anaerobic jars at 37°C until colonies were large enough to be counted.

(c) *Test plating media.*—Each of the 4 test media was prepared according to manufacturer's directions and was inoculated by using sterile glass rods to spread 0.1 mL portions of the appropriate dilutions on the surface of the agar of duplicate plates. Plates were incubated in anaerobic jars at 37°C until colonies were large enough to be counted.

Sample Preparation

Samples were prepared by blending, shaking, or diluting in sterile 0.1% peptone water. Minimal mixing is suggested even though *C. perfringens* is more air-tolerant than are most clostridia species (9). Serial decimal dilutions were prepared in sterile 0.1% peptone water. Tubes of milk medium were inoculated with 1.0 mL of the appropriate dilution and all tubes were incubated 16–18 h in a 45°C water bath.

Biochemical Tests

Standard biochemical tests were used to confirm *C. perfringens*. All test media were inoculated with 24 h pure cultures and incubated at 37°C (10).

Motility and nitrate reduction were tested by using a motility nitrate agar (11). This medium was inoculated by stabbing an actively growing culture to the bottom of the tube. For use with clostridia, the motility and nitrate medium performs best when prepared from fresh components. Nitrate reduction was tested within 18 h of inoculation.

Gelatin liquefaction and lactose fermentation were done by using a lactose-gelatin medium (11). An actively growing culture was inoculated to the bottom of the tube with a pipet.

Lipase and lecithinase production were tested by using an egg yolk agar of the following composition: peptone, 40 g; Na_2HPO_4, 5 g; NaCl, 2 g; $MgSO_4 \cdot 7\ H_2O$ (10% solution), 0.2 mL; glucose, 2 g; agar, 15 g; and distilled water, 1 L. The medium was autoclaved and then cooled to 45°C, and 10 mL egg yolk suspension (Difco) was added per 100 mL agar. Plates were poured, allowed to solidify, and inoculated by streaking. Lecithinase was detected by the zone of precipitate in the agar extending beyond the colonies. Lipase activity (iridescent "pearly" layer) characteristic for *C. botulinum* is not a typical reaction of *C. perfringens* and was negative in our tests.

Data Processing

The statistical package for the Social Sciences (SPSS) software as implicated on the CDC (Computer Data Corp.) system, University of Washington, was used for analyses of the raw data in Tables 1–3. One-way analysis of variance was used for the data in Table 2, and 2-way analysis of variance was used for data in Tables 1 and 3.

Results

The simple milk medium was compared with a nonselective enumeration procedure using blood agar. Dilutions of soil samples were inoculated into the milk medium MPN tubes and into blood agar by using the pour plate method. Colonies displaying beta haemolysis were picked

Table 2. Enumeration of low numbers of a known culture of *C. perfringens* from 5 replicate samples by each of the 5 test media

Replicate	Average No. of *C. perfringens*/0.1 mL by each medium[a]				
	A	B	C	D	E
1	4.3	4.0	3.0	4.0	5.0
2	4.3	5.0	8.5	5.5	2.5
3	4.3	7.0	7.0	5.0	4.0
4	7.5	2.0	2.5	5.5	6.5
5	1.5	4.5	8.0	12.5	4.0

[a] A = milk MPN method; B = TSC with egg yolk; C = TSC without egg yolk; D = SPS; E = TSN.

from the blood agar and tested for Gram reaction and biochemical tests (12). *Clostridium perfringens* enumerated by the milk MPN procedure were streaked on blood agar and subjected to the same biochemical tests. Average results for 5 soil samples were log 5.64 *C. perfringens*/g, using blood agar plates and log 5.54 *C. perfringens*/g, using the milk MPN procedure.

The milk MPN method was compared with 4 methods using pour plates (Table 1). Statistical analysis of the data (Table 4) shows that there were no significant differences between methods of counting; however, there was a significant difference between samples.

Bacteriological media for isolating specific bacteria must be reliable and reproducible. This is important in environmental, food, and clinical samples. To test the relative reproducibility and accuracy of the test media, we used a stock culture of *C. perfringens* from our laboratory collection as the inoculum for each of the 5 media. The numbers of cells were kept low so that the ability of each medium to detect fewer than 100 cells/0.1 mL sample could be evaluated. *Clostridium perfringens* were detected using the 5 test media (Table 2). Statistical analysis of the raw data showed that the low level counts obtained did not differ significantly.

Fifty organisms were isolated from each sludge, soil, and water sample by each of the 5 test media and were tested for confirmation as *C. perfringens*. These data for a total of 1250 isolates are shown in Table 3. When all 50 isolates were tested, the data were listed as numbers confirmed as *C. perfringens*. Numbers obtained varied between 39 and 50 organisms confirmed. Although the high confirmation rates were obtained with all 5 media, the milk MPN method gave the highest with confirmation for all isolates tested. Statistical analysis again showed that there were no significant differences between the media used.

Discussion

The use of pasteurized whole milk for the enumeration of *C. perfringens* was chosen because of the simplicity of the medium and its ready availability. The test is specific for *C. perfringens* because the organism grows rapidly at 45°C. The stormy fermentation reaction is the production of an acid curd with subsequent disruption of the curd by the large volumes of gas produced from fermentation of the lactose. It should be emphasized that a positive tube must contain all components which together make a stormy fermentation. In our tests with environmental samples, unidentified *Bacillus* species produced both a curd and a digestion on the surface of the milk. *Escherichia coli* produced a curd, digestion, and some gas bubbles in the

Table 3. Number of *C. perfringens* confirmed from the 50 organisms isolated from each sample by each medium

Sample No.	Sample	Medium and No. of organisms confirmed[a]				
		A	B	C	D	E
1	Sludge	50	50	40	50	49
2	Soil	50	47	50	39	38
3	Soil	50	50	43	48	40
4	Soil	50	42	47	43	50
5	Water	50	39	39	41	50

[a] A = milk MPN method; B = TSC with egg yolk; C = TSC without egg yolk; D = SPS; E = TSN.

Table 4. Analysis of variance for data in Tables 1, 2, and 3

Source of variation	Degrees of freedom	*F*-ratio	*F* probability
Table 1			
Between samples	4	171.58	0.001*
Between media	4	1.96	0.15
Table 2			
Between media	4	0.81	0.54
Table 3			
Between samples	4	0.68	0.62
Between media	4	1.21	0.35

* Significant difference between means.

milk. Both *Bacillus* and *E. coli* produce reactions similar to stormy fermentation which can be confusing to the untrained analyst, but their reactions are slower than for *C. perfringens* at 45°C. Therefore, best results are obtained when milk is incubated a maximum of 16–18 h, and then the reactions recorded as soon as the results can be evaluated.

A number of media and methods have been published for enumeration or identification of *C. perfringens*. Most probable number methods using litmus milk were reported by Dudley et al. (13), who used 37°C incubation, and by Erickson and Diebel (14), who incubated the litmus milk supplemented with 2 antibiotics and thioglycollate at 46–48°C.

Most of the agar media also incorporate antibiotics or other inhibitors which vary in their inhibition of *C. perfringens*. Although these media are very effective, as our comparative data show, for sludge, soil, and water samples, most have been tested and proven for food samples as well. The milk medium has been used with over 150 environmental samples but has not been adequately tested with food samples. Good results were obtained with a limited number of spices but these data are not included in this report. The medium has been tested with rat intestinal contents. In these studies (8, 15), more than 300 samples were analyzed for *C. perfringens* with subsequent confirmation using the biochemical tests given as well as growth with sulfite production on SPS and TSN agar.

Although the milk medium is used as part of the confirmation of *C. perfringens* (11), the added selectivity of a 45°C incubation temperature was adequate to confirm the presence of *C. perfringens*. Several species of *Clostridia* will grow (optimally) rapidly at 45°C, and several have been shown to produce a stormy fermentation. A combination of these 2 tests, stormy fermentation and rapid growth at 45°C, showed in the data presented here with soil, sludge, and water, and with rat gut contents (8, 15) that *C. perfringens* could be enumerated without confirmation. All *C. perfringens* isolated in this 2-year study were, in fact, confirmed.

The milk MPN method described here is an accurate and reproducible method that gives results comparable to other well known methods for enumerating *C. perfringens*. For samples for which the medium has not been tested, the use of confirmatory tests is suggested. This rapid MPN procedure has been used in our laboratory since 1976 (16).

The advantage of the milk method is the simplicity that results from the specificity of high temperatures and milk reactions. This method alone, without confirmatory biochemical tests, gave rapid, accurate counts of *C. perfringens* in the studies reported.

Acknowledgment

This work was supported by funds made available to the University of Washington by the Public Health Service Biomedical Research Support Grant.

References

(1) Angelotti, R., Hall, H. E., Foter, M. J., & Lewis, K. H. (1962) *Appl. Microbiol.* **10,** 193–199
(2) Shahidi, S. A., & Ferguson, A. R. (1971) *Appl. Microbiol.* **22,** 688–692
(3) Harmon, S. M., Kautter, D. A., & Peeler, J. T. (1971) *Appl. Microbiol.* **21,** 500–506
(4) Marshall, R. S., Steenbergen, J. F., & McClurg, L. S. (1965) *Appl. Microbiol.* **13,** 559–563
(5) Boyd, M. J., Logan, M. A., & Tyrell, A. A. (1948) *J. Biol. Chem.* **174,** 1013–1025
(6) Collee, J. G., Knowlden, J. A., & Hobbs, B. C. (1961) *J. Appl. Bacteriol.* **24,** 326–339
(7) Pappenheimer, A. M., & Shaskan, E. (1944) *J. Biol. Chem.* **155,** 265–275
(8) Wekell, M. M., Hartman, W. J., & Dong, F. M. (1980) *J. Nutr.* **110,** 2103–2108
(9) Smith, L. DS., & Holdeman, L. V. (1968) *The Pathogenic Anaerobic Bacteria*, Charles C Thomas,

Springfield, IL, 423 pp
(10) Matches, J. R., & Liston, J. (1973) in *Belle W. Baruch Library in Marine Science*, Vol. 1, *Estuarine Microbiol. Ecology*, L. H. Stevenson & R. R. Colwell (Eds), University of South Carolina Press, Columbia, SC, pp. 345–362
(11) *FDA Bacteriological Analytical Manual* (1978) 5th Ed., AOAC, Arlington, VA
(12) Cato, E. P., et al. (1970) *Outline of Clinical Methods in Anaerobic Bacteriology*, The Anaerobic Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA
(13) Dudley, D. J., Guentzel, M. N., Ibarra, M. J., Moore, B. E., & Sagik, B. P. (1980) *Appl. Environ. Microbiol.* **39,** 118–126
(14) Erickson, J. E., & Diebel, R. H. (1978) *Appl. Environ. Microbiol.* **36,** 567–571
(15) Hartman, W. T. (1980) M.S. Thesis, University of Washington, Seattle, WA
(16) Matches, J. R., & St. John, W. D. (1977) *Research in Fisheries*, March issue, University of Washington, Seattle, WA, pp. 56–57

New Method for Differentiating Members of the *Bacillus cereus* Group: Collaborative Study

STANLEY M. HARMON
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Collaborators: R. Bryant; D. Campbell; J. Chieu; L. English; J. Graham; G. Lancette; T. Latt; P. McLaughlin; M. Pratt; C. Noah; G. Nygaard; D. Radle: D. Staben; N. Stern; G. Varney

A collaborative study was conducted of a new method for differentiating members of the *Bacillus cereus* group. Using the new method, each of 14 collaborators identified 8 *Bacillus* cultures, which represented 3 biotypes of the *B. cereus* group. Each culture was tested for motility, hemolytic activity on trypticase-soy-sheep blood agar, and rhizoid growth on nutrient agar; carbol-fuchsin stain was used to determine the presence of protein toxin crystals. Cultures were identified as *B. cereus* (biotype 1), *B. cereus* var. *mycoides* (biotype 2), or *B. thuringiensis* (biotype 3). All collaborators correctly identified the unknown cultures and classified them correctly as to biotype. There were no statistically significant differences in the identification rates among the different laboratories. Additional tests by one participant on 5 strains of *Bacillus anthracis* showed that the new method is also adequate for differentiating *B. anthracis* from typically reacting strains of *B. cereus*. The method has been adopted interim official first action.

Although *Bacillus cereus* has been implicated in human food poisoning for many years, no reliable means are currently available for demonstrating the presence of either the diarrheal or emetic enterotoxins in foods (1). At present such foods are examined for the presence of large numbers of *B. cereus* and the organisms are identified as *B. cereus*. Microbiological procedures are needed to differentiate *B. cereus* from culturally similar organisms, including the rhizoid strains *B. cereus* var. *mycoides*, the insect pathogens presently classified collectively as *B. thuringiensis*, and the mammalian pathogen *B. anthracis*. The role in food poisoning, if any, of other biotypes of the *B. cereus* group has not been clearly established.

The taxonomy of the *B. cereus* group is very complex. Disagreement over the years about the taxonomic position of different species or varieties (2, 3) has been due in part to the difficulty of identifying biotypes and has led to further confusion about the relationship of culturally similar organisms (4). The rhizoid strains, for instance, occur frequently in soil and on vegetables such as grains and vegetable seeds or sprouts. In recent years, the insect pathogen *B. thuringiensis* has also been used extensively on food and forage crops for insect control and may conceivably be encountered in the examination of foods (5). *Bacillus anthracis* would present an extreme hazard if present in foods, but anthrax is rarely encountered in the food supply because diseased animals are condemned before entering the channels of commerce. Therefore, it is extremely unlikely that *B. anthracis* would be encountered in the routine examination of foods.

Because of these difficulties, no specific procedures for differentiating these organisms were recommended when the method for enumerating and confirming *B. cereus* was collaboratively studied and adopted official first action in October 1979 (6). Since that time, however, a new method for differentiating members of the *B. cereus* group has been developed by the Associate Referee and collaboratively studied. The purpose of the new method is to provide supplementary procedures for the identification of *B. cereus* isolated from foods by the official method (**46.A10**). The results of the collaborative study are the subject of this report.

Collaborative Study

Each of 14 collaborators received a complete set of instructions, a copy of the method to be studied, and data report forms. All materials except commercial trypticase-soy-sheep blood agar plates were purchased from the same suppliers. Culture media were prepared from commercially available dehydrated media or from ingredients specified in the method. Cultures were shipped by airmail and examined by the participants within 5 days of receipt. Collaborators were instructed to store the test cultures at

Received September 17, 1981. Accepted March 5, 1982.
The recommendation of the Associate Referee was approved by the General Referee and by Committee F and was adopted interim official first action by the Association.
This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

0004-5756/82/6505-1134-06$01.00

4°C until examined and to begin the analysis on a specific date if possible. The results were reported to the Associate Referee within 1 month after cultures were received.

In phase 1 of the study, each participant examined known cultures of the *B. cereus* group (including 1 emetic and 1 diarrheal strain of *B. cereus*, 2 rhizoid strains of *B. cereus* var. *mycoides*, and 2 strains of the insect pathogen *B. thuringiensis*) to become familiar with the reactions of different biotypes before attempting to identify unknown cultures. In phase 2 of the study, each participant examined 8 unknown cultures, which represented 3 biotypes of the *B. cereus* group. These cultures included duplicate cultures of an emetic *B. cereus* (biotype 1) strain F4810/72 (Serotype 1) and a diarrheal strain F4433/73 (Serotype 2) obtained from R. Gilbert (7), 2 different strains of *B. cereus* var. *mycoides* (biotype 2), and 2 strains of the insect pathogen *B. thuringiensis* (biotype 3). One laboratory also used the method to examine 5 strains of the mammalian pathogen *B. anthracis*. The unknown cultures were subcultured on nutrient agar slants and coded with an identification letter and number combination to maintain anonymity until all collaborative results were received.

Differentiation of Members of *Bacillus cereus* Group

Typical strains of *B. cereus* can be differentiated from other members of *B. cereus* group including: (*1*) insect pathogen *B. thuringiensis*, (*2*) mammalian pathogen *B. anthracis*, and (*3*) rhizoid strains *B. cereus* var. *mycoides*.

Apparatus

(**a**) *Staining rack.*—Rack must be accessible from below for heating slides.

(**b**) *Inoculating loops.*—One each, 26 gage nichrome wire with loop 2 mm id and one 24 gage nichrome wire loop 3 mm id.

Media and Reagents

(**a**) *Mannitol-egg yolk-polymyxin (MYP) agar.*—1.0 g beef ext, 10.0 g peptone, 10.0 g D-mannitol, 10.0 g NaCl, 0.025 g phenol red (as soln), and 15.0 g agar dild to 900 mL with H_2O. Adjust to pH 7.2 ± 0.1, heat to dissolve, and dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Cool to 50° in H_2O bath and add 12.5 mL sterile 50% egg yolk emulsion (**b**) and 2.5 mL polymyxin B soln contg 10 000 units per mL (if available) to each 225 mL medium. (Addn of polymyxin B soln is optional when medium is to be used for testing reactions of pure cultures.) Mix well and dispense 18 mL portions into 100 × 15 mm sterile petri dishes. Dry plates 24 h at room temp. before use. (Dehydrated mannitol-egg yolk-polymyxin (MYP) agar contg 50% egg yolk enrichment is satisfactory.)

(**b**) *Egg yolk emulsion.*—50%. Wash fresh eggs with stiff brush and drain. Soak 1 h in 70% alcohol. Aseptically remove yolk and mix (1 + 1) with sterile 0.85% NaCl soln. (50% egg yolk enrichment is satisfactory.)

(c) *Nutrient agar slants and plates.*—3.0 g beef ext, 5.0 g peptone, and 15.0 g agar dild to 1 L with H_2O (dehydrated nutrient agar is satisfactory). Heat to dissolve, and dispense 6.5 mL portions into 125 × 16 mm screw-cap tubes. Autoclave 15 min at 121° and slant tubes until medium solidifies. Final pH 6.8 ± 0.2. For plates, dispense 100-500 mL portions in bottles or flasks and autoclave 15 min at 121°. Cool to 50° in H_2O bath and dispense 18-20 mL portions in 100 × 15 mm sterile petri dishes. Dry plates 24-48 h at room temp. before use.

(d) *Motility medium.*—10.0 g trypticase, 2.5 g yeast ext, 5.0 g dextrose, 2.5 g Na_2HPO_4, and 3.0 g agar dild to 1 L with H_2O. Heat to dissolve. Dispense 2 mL portions into 13 × 100 mm tubes, and autoclave 10 min at 121°. Final pH 7.4 ± 0.2. Alternatively, dispense 100 mL amts in 150 mL bottles and autoclave 15 min at 121°. Cool at 50° and aseptically dispense 2 mL into sterile 13 × 100 mm tubes. For best results, store at room temp. 2-4 days before use to prevent growth along side of medium.

(**e**) *Trypticase-soy-sheep blood (TSSB) agar.*—Dil. 15.0 g trypticase, 5.0 g phytone peptone, 5.0 g NaCl, and 15.0 g agar to 1 L with H_2O. Adjust pH to 7.0 ± 0.2. Heat to boiling to dissolve, and dispense 100-500 mL portions in bottles or flasks. Autoclave 15 min at 121° and cool to 48° in H_2O bath. Add 5 mL sterile defibrinated sheep blood per 100 mL medium. Mix well, and dispense 18-20 mL portions into 100 × 15 mm petri dishes. (Trypticase-soy or tryptic-soy agar plates contg 5% sheep blood are satisfactory.)

(**f**) *Basic fuchsin stain.*—Dissolve 0.5 g basic fuchsin in 20 mL alcohol and dil. to 100 mL with H_2O. Filter soln if necessary thru fine paper to remove excess dye particles. Store in tightly stoppered container. (TB Carbol-fuchsin ZN stain is satisfactory.)

(**g**) *Butterfield's buffered phosphate diluent.*—(*1*) *Stock soln.*—Dissolve 34.0 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with ca 175 mL 1N NaOH, and dil. to 1 L with H_2O. Store in refrigerator.

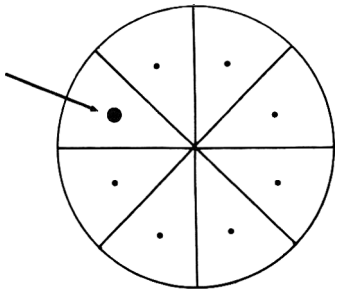


Figure 1. Diagram of template for marking and inoculating *B. cereus* confirmatory plates. Each section is labeled and inoculated in the center, as indicated by arrow.

(2) *Diluent.*—Dilute 1.25 mL stock soln to 1 L with H_2O. Prep. 90 ± 1 mL diln blanks with this soln and autoclave 15 min at 121°. Dispense 0.5 mL portions sterile diluent into sterile 13 × 100 mm tubes for preparing suspension of cultures to be tested.

(**h**) *Methanol fixative.*—Dispense undild methanol in plastic squeeze bottle for use in fixing slides.

Differential Tests

(**a**) *Preparing test inoculum.* Inoculate sep. nutrient agar slants with each culture to be tested. Incubate slants 18–24 h at 30° and transfer 3 mm loopful of culture from each slant to 100 × 13 mm tube contg 0.5 mL sterile phosphate buffered diluent. Suspend culture in diluent with vortex mixer. Alternatively, inoculate 5 mL trypticase-soy broth and incubate tubes 18 h at 30°. Mix culture well and use for performing differential tests. Latter procedure is preferred for rhizoid strains and other strains which do not disperse well in phosphate buffer.

(b) *Reaction on MYP agar.*—Mark bottom of MYP agar plate into 6–8 equal segments with black felt pen as indicated in Figure 1 and label each section. Place plate in upright position on piece of white paper and inoculate one or more of the prelabeled sections by gently touching surface of agar with 2 mm loopful of culture. Let inoculum be absorbed and incubate plates in upright position 24–48 h at 30–35°. Check for lecithinase production as indicated by zone of ppt surrounding growth. Mannitol fermentation is neg. if growth and surrounding medium are eosin pink. These reactions should be observed with all organisms of *B. cereus* group except rare lecithinase-neg. variants.

(c) *Motility tests.*—Inoculate BC motility medium by stabbing down center with 3 mL loopful of culture. Incubate 18–20 h at 30° and examine for type of growth along stab. Motile strains produce diffuse growth into medium away from stab. Nonmotile strains except *B. cereus* var. *mycoides* grow only in and along stab. Strains of *B. cereus* var. *mycoides* often produce "fuzzy" growth in semisolic media resulting from cellular expansion but are not motile by means of flagella. Recheck doubtful results by alternative microscopic motility test as follows: Add 0.2 mL sterile H_2O to nutrient agar slant and inoculate with 3 mm loopful of culture. Incubate slant 6–8 h at 30°, and mix small loopful of liq. culture from base of slant with drop of sterile H_2O on microscope slide. Apply cover glass and examine immediately for signs of motility. *B. cereus* and *B. thuringiensis* cultures are usually actively motile by means of peritrichous flagella. *B. anthracis* and typically rhizoid strains of *B. cereus* var. *mycoides* are nonmotile.

(**d**) *Rhizoid growth.*—Inoculate predried nutrient agar plate by touching medium surface near center with 2 mm loopful of culture. Let inoculum be absorbed, and incubate plate in upright position 24–48 h at 30°. Check plate for rhizoid growth characterized by root or hairlike structures which may extend several cm from point of inoculation. Many *B. cereus* strains produce rough irregular colonies that should not be confused with rhizoid growth. This property is characteristic only of strains which are classified as *B. cereus* var. *mycoides*.

(**e**) *Hemolytic activity.*—Mark bottom of trypticase-soy-sheep blood agar plate into 6–8 equal segments (see Figure 1) with black felt marking pen. Label each segment and inoculate one or more segments near center by gently touching agar surface with 2 mm loopful of culture. Let inoculum be absorbed, and incubate plates 24 h at 30–32°. Check plates for hemolytic activity as indicated by 2–4 mm zone of complete (beta) hemolysis surrounding growth. *B. cereus* is usually strongly hemolytic, whereas *B. thuringiensis* and *B. cereus* var. *mycoides* are often weakly hemolytic and produce complete hemolysis only underneath colonies. *B. anthracis* is usually nonhemolytic after 24 h of incubation. *Caution:* Nonmotile, nonhemolytic cultures could be *B. anthracis*. See precautions under *interpreting test results*, (**g**).

(**f**) *Detection of toxin crystals.*—Inoculate nutrient agar slant with loopful of culture. Incubate slant 24 h at 30° and hold at room temp. 2–3 days. Make smear on microscope slide with sterile H_2O. Air-dry and briefly heat-fix by passing slide slowly over burner flame; let cool,

and place slide on staining rack. Flood slide with methanol, wait 30 s, and pour off methanol. Dry thoroughly by passing through burner flame. Return slide to staining rack, and flood completely with 0.5% aq. soln of basic fuchsin or TB Carbol–fuchsin ZN stain. Heat slide gently from below with micro burner until steam is seen. Wait 1–2 min and repeat this step. Let stand 30 s, pour off stain, and rinse slide thoroughly in 1 L clean tap H_2O. Dry slide without blotting and examine microscopically under oil immersion for presence of free spores and darkly stained tetragonal (diamond-shaped) toxin crystals. Free toxin crystals are usually abundant after 3 days but will not be detectable unless sporangia have lysed. Therefore, if free spores are not seen, leave cultures at room temp. for a few more days and repeat test. *B. thuringiensis* produces protein toxin crystals that usually can be detected by staining, but are not produced by other members of *B. cereus* group.

(g) *Interpreting test results.*—On basis of test results, identify as *B. cereus* those isolates which are actively motile, strongly hemolytic, and do not produce rhizoid growth or protein toxin crystals. Nonmotile strains of *B. cereus* may be encountered and a few are weakly hemolytic. These strains can be differentiated from *B. anthracis* by their resistance to penicillin and to gamma bacteriophage. *Caution:* Nonmotile, nonhemolytic strains could be *B. anthracis*, and should be handled with special care and submitted to pathology laboratory such as Centers for Disease Control for identification or destroyed by autoclaving. Noncrystalliferous variants of *B. thuringiensis* and nonrhizoid strains derived from *B. cereus* var. *mycoides* cannot be differentiated from *B. cereus* by tests described.

Results and Discussion

All 14 collaborators correctly identified all 8 of the unknown *Bacillus* cultures. Identical results were reported with the duplicate culture of both the emetic and diarrheal strains of *B. cereus* and with 2 different strains of *B. thuringiensis* and *B. cereus* var. *mycoides*. The only differences were a matter of intensity of positive reactions. One participant also examined 5 strains of *B. anthracis* and compared the results with those of the 3 strains of *B. cereus* which were collaboratively studied. The results indicated that the new method is suitable for differentiating the typically reacting strains of *B. cereus* from the other biotypes of the *B. cereus* group, including the rhizoid strains presently classified as *B. cereus* var. *mycoides*, the insect pathogen *B. thuringiensis*, and the mammalian pathogen *B. anthracis*. Individual collaborative results, which are summarized in Table 1, varied slightly, particularly the hemolytic and lecithinase activities of *B. thuringiensis* and *B. cereus* var. *mycoides*. The hemolytic activity of these strains is usually weak and shows greater variation than that of the typical *B. cereus* strains. It was concluded, however, that the variations were due mostly to the analysts' lack of experience in interpreting results. In any case, these discrepancies did not prevent any analyst from correctly identifying all 8 of the unknown cultures.

Two participants reported some difficulty in interpreting results of the motility tests and 2 had difficulty recognizing the toxin crystals produced by *B. thuringiensis*. Because many microbiologists are unfamiliar with the cultural reactions of the different biotypes, they should include at least 1 culture of each biotype (except *B. anthracis*) as a control to become familiar with the reactions before attempting to identify unknown cultures. The analyst who examined the *B. anthracis* cultures also felt that unless *B. cereus* cultures are actively motile and strongly hemolytic, they cannot be distinguished from *B. anthracis* by this method. The Associate Referee concurs and recommends that cultures suspected to be *B. anthracis* be submitted to a pathology laboratory with proper facilities and personnel for identifying this organism.

The collaborative data were analyzed statistically using a chi square test to determine any significant differences in the identification rates of *B. cereus* or of the other biotypes. Since no cultures were incorrectly identified by the collaborators, there were no significant differences in the identification rates of either the *B. cereus* cultures or the other biotypes ($P > 0.25$). Qualitatively, the new method appears to be very reliable for differentiating the *B. cereus* biotypes tested; all collaborators correctly classified the 8 test cultures, including those of biotypes other than *B. cereus*.

Recommendation

Based on the results of the collaborative study, it is recommended that the official method of analysis for enumeration of *B. cereus* in food be amended to include the differential tests described. Such a change would greatly improve the official method by providing a simple and reliable means for identifying isolates from foods as *B. cereus*.

Table 1. Cultural reactions of 4 different biotypes of the *B. cereus* group: summary of collaborative results

Species tested	Strain(s)	Reaction on MYP agar		Motility		Rhizoid growth on nutrient agar plate	Hemolysis[a]		Toxin crystals present after 4 days
		Lecithinase	Mannitol	Motility medium	Slide test		Complete	Partial	
B. cereus	F4433/73 diarrheal	+	–	+	+	–	3 +[b]	+	–
B. cereus	F4810/72 emetic	+	–	+	+	–	2+	+	–
B. cereus var. *mycoides*	3	(+)	–	–	–	+[c]	(+)[d]	2 – 3+	–
B. cereus var. *mycoides*	4	(+)	–	–	–	+	(+)	2 – 3+	–
B. cereus var. *mycoides*	NRZ non-rhizoid variant	+	–	–	–	–	1+ – 2 +[c]	2+	–
B. thuringiensis	A – 1	+	–	+	+	–	1+	2 – 3+	+[c]
B thuringiensis	W	+	–	+	+	–	1+	2 – 3+	+
B. anthracis[e]	5 strains	+	–	–[c]	–	–	–[c]	–[c]	–

[a] On trypticase–soy–sheep blood agar plates (range of results reported).
[b] Width of hemolysis zone (mm) measured from edge of growth.
[c] Critical characteristic for identification of biotype.
[d] Complete hemolysis only underneath colony.
[e] *B. anthracis* strains CDC 1014, 1288, 1289, 1617, and 3311, tested by only 1 collaborator.

Acknowledgments

The Associate Referee gratefully acknowledges Foster McClure, Division of Mathematics, Food and Drug Administration (FDA), Washington, DC for compiling the statistical data, and the following analysts for their participation in the study: Raymond Bryant, California Dept of Health Services, Berkeley, CA; Douglas Campbell, U.S. Dept of Agriculture, Beltsville, MD; Joseph Chieu, U.S. Dept of Agriculture, Athens, GA; Linda English and Thomas Latt, FDA, Baltimore, MD; Janet Graham, Alabama Dept of Public Health, Montgomery, AL; Gayle A. Lancette, FDA, Minneapolis, MN; Philip E. McLaughlin, FDA, New York, NY; Mark Pratt, U.S. Dept of Agriculture, St. Louis, MO; Charles W. Noah, FDA, Dallas, TX; David C. Radle, FDA, Cincinnati, OH; David Staben, FDA, Denver, CO; Norman Stern, U.S. Dept of Agriculture, Beltsville, MD; George Varney, FDA, Boston, MA.

Special thanks are extended to Genevieve Nygaard of the Special Bacteriology Unit, California Dept of Health Services, Berkeley, CA, for performing the analyses with *B. anthracis*.

References

(1) Gilbert, R. J. (1979) in *Food-Borne Infections and Intoxications*, 2nd Ed., H. Riemann & F. L. Bryan (Eds), Academic Press, New York, NY

(2) Brown, E. R., Moody, M. D., Treece, E. L., & Smith, C. W. (1958) *J. Bacteriol.* 75, 499–509

(3) Gordon, R. E., Haynes, W. C., & Pang, C. H. N. (1973) *The Genus Bacillus*, Agriculture Handbook No. 427, U.S. Department of Agriculture, Washington, DC, pp. 23–24

(4) Kreig, A. (1969) *J. Invertebrate Pathol.* 15, 313–320

(5) Falcon, L. A. (1971) *Microbial Control of Insects and Mites*, H. D. Burges & N. W. Hussey (Eds.), Academic Press, New York, NY

(6) Lancette, G. A., & Harmon, S. M. (1980) *J. Assoc. Off. Anal. Chem.* 63, 581–586

(7) Taylor, A. J., & Gilbert, R. J. (1975) *J. Med. Microbiol.* 8, 543–550

METALS AND OTHER ELEMENTS

Recovery of Endogenous Selenium from Fish Tissues by Open System Dry Ashing

THOMAS W. MAY
U.S. Fish and Wildlife Service, Columbia National Fisheries Research Laboratory, Route 1, Columbia, MO 65201

Quantitative recovery of endogenous selenium from fish tissues following dry ashing techniques has not been confirmed. An open system dry ashing procedure, using oxidative fusion with $Mg(NO_3)_2 \cdot 6H_2O$ in the presence of MgO, was tested for recovery of selenium from National Bureau of Standards (NBS) Reference Materials (tuna and oyster) and selected fish tissues. Recovery after dry ashing and hydride analysis was complete from NBS Bovine Liver, Tuna, and Oyster Tissues, as well as from Food and Drug Administration cod, haddock, perch, and flounder research materials. Endogenous selenium, injected into rainbow trout (*Salmo gairdneri*) as ^{75}Se, was recovered after ashing from liver, ovaries, gastrointestinal tract, muscle, bile, and carcass, 3 and 10 days post-injection. Results indicate that open vessel oxidative fusion is a rapid, simple technique applicable to quantitative recovery of selenium from organoselenium compounds that exist in various aquatic species. The diluted digestate is readily amenable to conventional hydride generation analysis.

Perhaps the most widely used method for digesting biological materials for the determination of selenium involves wet digestion with acid mixtures. Mixtures of HNO_3–H_2SO_4 and HNO_3–$HClO_4$ have been used with some success (1, 2), but now the most widely accepted acid mixture is HNO_3–$HClO_4$–H_2SO_4 in various combinations (3–7). Besides being time consuming and requiring constant operator attention and specialized equipment ($HClO_4$ hoods, fume traps, scrubbing towers, etc.), digestion procedures with this combination of acids have led to poor selenium recoveries (7). Problems can occur from excessive heating that causes extremely rapid evolution of oxides of nitrogen; sample loss from excessive foaming; sample charring; an $HClO_4$ reaction that is too vigorous; and non-uniformity of heating from aluminum blocks, hot plates, and individual micro-Kjeldahl burner elements. Constant operator attention is often required to control many of these problems. Dry ashing methods offer the advantages of simplicity of equipment, high sample throughput, little operator attention, and less variable heating environments; therefore an investigation of the status of dry ashing techniques for selenium recovery seemed appropriate.

A review of the literature revealed few references that report selenium recovery values from open system dry ashing of organic materials. Gorsuch's extensive radiochemical recovery work (1) reported virtually complete tracer ^{75}Se loss from cocoa when the combined wet and dry ashing method of Middleton and Stuckey (8) was used. Consequently, Gorsuch did not investigate recoveries of selenium by other dry ashing procedures. Complete recovery by low temperature plasma ashing is questionable. Gleit and Holland (9) recovered 99% of ^{75}Se from alfalfa by low temperature plasma ashing, but others reported that the recovery of inorganic selenium (H_2SeO_3) from cellulose depended on the presence of other metals and radiofrequency coil power setting (10). One investigator (11) claimed successful dry ashing of fish tissues for selenium when $Mg(NO_3)_2$ and a preliminary treatment with HNO_3 were used, but no data for selenium recovery were presented. Other workers reported some losses during simple oven drying of plant and animal tissue at 100 and 120°C (12–14). Such information, along with Gorsuch's generalization that dry ashing methods for selenium cause serious losses unless they are carried out in closed systems (15), probably served as a deterrent to the study of dry ashing methods after 1970.

Arsenic is frequently determined along with selenium in the same sample, so a common decomposition technique would be desirable. There was significant work in the early and mid 1970s on open system dry ashing techniques for the determination of arsenic in plant and animal tissues (16–19), but selenium recoveries by these procedures were apparently either not attempted or not reported. When recovery data from open

Received January 15, 1982. Accepted March 10, 1982.

0004-5756/82/6505-1140-06$01.00

system dry ashing was reported, it was often based on spiking samples with inorganic selenium (20, 21), which does not test recovery of the specific organoselenium compounds actually present. Endogenous selenium recovery data, when it was reported, was based on NBS Orchard Leaves (22, 23), NBS Bovine Liver (22, 23), and Kodak gelatin reference material (23). Tam and Lacroix (24), using an arsenic dry ashing procedure (25), were the first to demonstrate that endogenous selenium could be quantitatively recovered from animal tissues other than NBS Bovine Liver.

A review of the literature confirmed that quantitative recovery of endogenous selenium from animal tissues following open system dry ashing with $Mg(NO_3)_2$ has been demonstrated only for NBS Bovine Liver (22, 23) and specific rat tissues (21, 24); for fish tissues, the technique lacks supporting recovery data. Little is known about the specific chemical forms of selenium in fish (26), and thus even less is known about how these specific forms behave under the rigorous conditions of dry ashing. With present-day emphasis on chemical speciation and identification of the biological forms of elements, the analyst must assume, until it is known otherwise, that there are differences in the chemical forms of selenium in bovine, mammalian, and aquatic vertebrates. It is for this reason, as well as the already mentioned advantages over wet ashing, that the applicability of open vessel dry ashing of fish tissues for subsequent selenium determination was investigated.

Experimental

Sample Selection

To test for Se recovery, only samples with known Se concentrations were considered. Samples of fish tissue internal standards, which were originally prepared from ground and lyophilized fillets of cod, flounder, perch, and haddock, were obtained from the Food and Drug Administration (FDA). These samples were deemed particularly valuable because the matrix was fish, and considerable Se concentration data existed for each, confirmed by totally independent techniques (4). Selected NBS Reference and Research Materials were NBS RM 50 (Albacore Tuna), NBS 1577 (Bovine Liver), and NBS 1566 (Oyster Tissue).

Reagents

Several grades of $Mg(NO_3)_2 \cdot 6H_2O$ were investigated for Se background levels. Essentially no difference in Se background was apparent between 2 of the grades tested: the ultrapure $Mg(NO_3)_2 \cdot 6H_2O$ (Alfa Products) and MgO (Spex MG70-2) and analytical reagent $Mg(NO_3)_2 \cdot 6H_2O$ (Mallinckrodt) and MgO (Alfa Products, 99.5%, 325 mesh). Therefore, analytical reagent grade materials were used for all ashing. The ashing aid was prepared by dissolving 80 g $Mg(NO_3)_2 \cdot 6H_2O$ in 200 mL deionized water (15–18 megohm-cm specific resistivity) and adding 10 g MgO powder. Methanol was MCB OmniSolv, glass distilled. Reductant solution consisted of 3% $NaBH_4$ (98%, Alfa Products, 10/32 in. pellets), with 1% NaOH (MCB, ACS grade) added as a stabilizer.

Dry Ashing Procedure

The procedure was a modification of the dry ashing method of Tam and Lacroix (24). About 0.5 g lyophilized tissue was wetted with methanol in a 100 mL Pyrex beaker with watch glass. Ten mL ashing aid was added, and the mixture was dried overnight in a 110°C oven. The dried sample was then transferred to a cold muffle furnace (Thermolyne Model F-A1740) and ashed overnight at 500°C. Accuracy of the drying oven and furnace was verified with a calibrated pyrometer. Ashed residue was dissolved by adding 10 mL deionized water, followed by 15 mL concentrated HCl. Se^{+6} was quantitatively reduced to Se^{+4} by heating the sample on a 400°F hot plate just until the ashed residue was solubilized. After beaker contents had cooled, they were transferred to a preleached (27) conventional polyethylene bottle (28) and diluted to a final volume of 100 mL with deionized water.

Instrumentation

Se was determined by hydride generation using a Perkin-Elmer MHS-10 hydride system interfaced to a P-E Model 5000 atomic absorption spectrophotometer. Instrumental conditions and related information were as follows: acid matrix, 15% HCl; λ, 196 nm; EDL lamp, 6 watts; slit width, 0.7 nm; signal mode, concentration and peak height; integration time, 15 s; range of standard curve, 0–14 ppb. Ten mL sample or an appropriate dilution was placed in a polypropylene flask and reacted with 3% $NaBH_4$–1% NaOH solution. Hydrogen selenide generated was flushed with argon into an open-ended quartz cell heated with an air–acetylene flame. Automatic curvature correction, in addition to concentration calibration, was used to establish and maintain working curve linearity up to 14 ppb (standard concentration). Concentration

readouts were the average of duplicate determinations and were recorded by a P-E PRS-10 printer sequencer. Baseline drift and argon purge efficiency were monitored by tracings from a P-E Model 056 recorder. The method of additions was used where necessary to correct for matrix interferences.

Neutron Activation Analysis

The FDA fish samples were prepared and analyzed in 1973–74, so a current analysis by neutron activation (NAA) was necessary to verify reported Se concentrations (4) after a 7–8 year storage period. Neutron activation analysis was conducted at the University of Michigan Ford Nuclear Reactor. About 200 mg lyophilized fish was loaded into high purity fused silica tubes and sealed. Samples were irradiated for 40, 2 megawatt hours at an approximate thermal neutron flux of 1.5×10^{13} n/cm^2-sec. During irradiation, samples were rotated in a silicon bucket to ensure uniform neutron dose. Four weeks after irradiation, samples were counted with a lithium germanium detector (FWAM 2.3 KeV) having an efficiency of 20% for ^{60}Co gamma rays at 25 cm compared with that of 3 × 3 in. NaI detector.

Endogenous ^{75}Se Recovery

^{75}Se (4 mCi, specific activity 49.5 mCi/mg) was obtained from New England Nuclear as selenious acid (selenate free) in 0.5M HCl (0.1 mL); the radionuclide was diluted to 4 mL with physiological saline (0.85% NaCl). Each of 8 rainbow trout, *Salmo gairdneri*, 200–250 g, received intracoelomic injections of 0.5 mCi ^{75}Se. Feeding was terminated 24 h before injection, and the fish were sacrificed at 3 and 10 days post-injection. Composite samples (each 3–5 individuals) of liver, bile, ovary, gastrointestinal tract (with associated mesenteries), muscle (with skin), and carcass were collected. The carcass consisted of fish remains after removal of the named tissues—primarily head, gills, heart, kidney, and backbone. A composite sample tissue was reduced in size with stainless steel scalpels and placed in a 2000 mL glass vertical flute flask. Deionized water was added and the mixture was blended at 10 000 rpm with a Tekmar SD-45 homogenizer equipped with a G-450 generator head and Teflon bearing shaft (SD-45N). Three aliquots of homogenate were placed in Beckman scintillation vials (Bio Vials) to serve as controls. Tissue homogenate aliquots for dry ashing ranged from 200 μL (bile) to 10 mL (tissues) and were prepared in triplicate. After dry ashing, portions of the dissolved and diluted digestate, equal in volume to controls, were selected for counting. ^{75}Se radioactivity was measured with a Beckman Gamma 4000 counting system (single channel) equipped with a 3 in. NaI crystal doped with TlI. For controls and sample, the entire spectrum of ^{75}Se gamma pulses was counted to <1% relative standard deviation (RSD) (29). The ratio of gross sample counting rate (counts per unit time) to background counting rate for the lowest activity material was >40. Average Se radioactivity of 3 ashed aliquots was compared with that of the unashed homogenates for calculation of ^{75}Se recovery.

Results and Discussion

The character of the ash residue is that of a white fluffy mass occasionally containing trapped gas pockets that produce a puffed appearance. Some samples, particularly liver, entrap excessive gas during oven drying. Dried liver samples of 0.5 g or more can entrap enough gas to foam out of a 100 mL beaker during oven drying with ashing aid to 110°C. This situation can be controlled by adding 100–300 μL Dow Corning DB 110A antifoam emulsion, which reduces gas entrapment by 50–75% without significantly contributing to selenium background.

MgO, with a melting point of 2850°C, is unreactive during ashing at 500°C. The powder is only slightly soluble in water, with which it combines to form $Mg(OH)_2$ (30). The pH of a 40% (w/w) $Mg(NO_3)_2{\cdot}6H_2O$ solution is about 3.8. Saturation with MgO imparts an alkaline reaction to the mixture, increasing pH to 8.8. A secondary function of MgO is the formation of an inert layer on the bottom of the ashing vessel that, upon oven drying, effectively separates the sample from contact with the ashing vessel (15). Decreased proximity with the material of the ashing vessel reduces the likelihood of vessel retention of selenium. Sample residue after ashing is also alkaline (15), being primarily composed of MgO (23). Thus, alkalinity is maintained through oven drying and ash formation. Alkaline and oxidizing conditions favor the stability of Se^{+6} (31), and undoubtedly contribute to its survival through the rigorous oxidative fusion conditions of dry ashing with $Mg(NO_3)_2{\cdot}6H_2O$.

Values generated from atomic absorption (AA)/hydride analyses of NBS materials for selenium (Table 1) agree well with certified or "most probable" concentration levels reported by NBS. Method of additions was used to correct

Table 1. Results of hydride generation analysis of NBS tissues for selenium after open system dry ashing

Material[a]	n[b]	$\bar{X} \pm$ SD	Reported value[c]
Bovine Liver	6	1.1 ± 0.2	1.1 ± 0.1
Albacore Tuna	6	3.5 ± 0.2	3.6 ± 0.4
Oyster Tissue	6	1.8 ± 0.2	2.1 ± 0.5

[a] Not corrected for moisture, which, in desiccated atmosphere, ranges from 1 to 3%.
[b] n = number of replicate aliquots.
[c] 95% tolerance limits (32).

for substantial matrix interferences present as signal suppression during the hydride analysis of NBS Bovine Liver. Such suppression was not apparent for NBS Tuna or Oyster.

In the analysis of FDA samples (Table 2), replicates of the same sample by AA/hydride and NAA are less variable in 1980 than in 1974, partly because of the way the samples were analyzed. For example, AA/hydride results for 1980 were based on 6 replicates all analyzed at one time, whereas those from 1974 were based on 5 replicate analyses gathered over 4 months (4), thus incorporating added variability in sample digestion and instrumentation. Other factors reducing variability in 1980 may be improvements in hydride instrumentation and the added experience of nuclear reactor laboratories in the preparation and irradiation of fish tissues. AA/hydride values from studies in 1974 appear high compared with those for 1980 and with those from NAA and fluorometry analyses done in the same period. Results obtained in 1980 by AA/hydride and NAA agree well, and confirm that the samples were not affected by long-term storage.

Mean recoveries of endogenous ^{75}Se at 3 and 10 days post-injection for 6 different dissected fish tissues ranged from 91 to 108% (Table 3). Overall average recovery based on 18 sample aliquots was about 102 and 95% for both time periods; these results suggest virtual recovery of incorporated selenium when the described open vessel dry ashing procedure is used. ^{75}Se was apparently incorporated into the tissues by normal or nearly normal biological processes. Although the tissue chemical form of selenium may not be identical with that obtained from chronic environmental exposure, the results are encouraging and suggest that open vessel oxidative fusion does apply for quantitative recovery of environmentally incorporated selenium. To test recovery of exogenously added selenium, inorganic selenium as $H_2{}^{75}SeO_3$ was spiked into 16 fish tissue homogenate samples (whole body) before oven drying; recovery after dry ashing was 98 ± 4% ($\bar{X} \pm$ SD).

Many methods have been used for the separation of selenium from organic materials. Recovery from plant and animal tissues has posed problems, almost irrespective of the decomposition method attempted. Losses, or poor recoveries, have been reported from use of the wet acid mixtures HNO_3–H_2SO_4 (1, 33) and HNO_3–$HClO_4$–H_2SO_4 (7), combined wet and dry ashing methods (1, 15), and low temperature plasma ashing (10, 21). Various books and reviews refer to organic material dry ashing for selenium recovery as causing serious losses (15) or as being unreliable (34) unless carried out in closed systems. Van Loon (35) stated that chalconides (Ge, Sb, Bi, As, Se) will most likely be lost in varying amounts during dry ashing of plant materials. Bem (36) attributed the widespread use of wet oxidation methods to selenium losses in the dry ashing method and limitation of sample size in the oxygen flask technique. Such published reports of selenium losses from dry ashing of

Table 2. Results of analysis of FDA materials for selenium: comparison of independent method results over 2 time periods (values expressed as $\bar{X} \pm$ SD (μg/g dry weight))

Material	Dry ashing, AA/hydride[a] 1980	NAA[b] 1980	HNO_3–$HClO_4$–H_2SO_4,[c] AA/hydride 1974	Fluorometry[d] 1974	NAA[e] 1974
Cod	1.14 ± 0.24	1.26 ± 0.11	1.4 ± 0.4	1.2	1.2 ± 0.2
Haddock	1.08 ± 0.15	1.23 ± 0.04	1.6 ± 0.4	1.6	1.6 ± 0.4
Perch	2.05 ± 0.13	2.14 ± 0.04	2.6 ± 0.3	2.3	2.2 ± 0.2
Flounder	0.79 ± 0.03	0.87 ± 0.05	1.2 ± 0.2	0.9	0.9 ± 0.3

[a] Based on 6 replicate analyses, all conducted at the same time.
[b] Based on 5 replicate analyses, all conducted at the same time.
[c] Based on 5 replicate analyses over a 4 month period (4).
[d] Single analysis (4).
[e] Based on 3–5 replicate analyses, time format not reported (4).

Table 3. Recovery of incorporated ^{75}Se from fish tissues at 3 and 10 days post-injection by open vessel dry ashing

Tissue	% Recovery ± SD	
	3 days	10 days
Liver	104 ± 2[a]	97 ± 3
Ovary	108 ± 2	97 ± 2
Gastrointestinal tract	99 ± 1	92 ± 1
Muscle	101 ± 1	91 ± 6
Bile	96 ± 1	99 ± 1
Carcass	105 ± 2	96 ± 7
Overall average[b]	102 ± 4	95 ± 5

[a] Based on 3 replicate analyses.
[b] Based on 18 individual analyses, 3 of each tissue type.

organic materials should serve as a warning to the analyst that the technique cannot be generalized to be acceptable for all animal tissues or food products. Whereas quantitative recovery of endogenous selenium from bovine liver and orchard leaves following dry ashing has been demonstrated (22, 23), virtually the same procedure resulted in 20% losses of selenium from rat feces and urine (25).

It is apparent that generalizations about dry ashing are inaccurate, because selenium recovery depends on the organic matrix involved and the specific type of dry ashing procedure used. "Dry ashing" itself is a general term that includes several different open and closed system techniques (15). Open system procedures include sample ashing with (oxidative fusion) and without ashing aids, and low-temperature plasma ashing. Closed system methods include conventional combustion trains, oxygen flask combustion, oxygen bombs, and oxidative fusion. Thus, efficiency of selenium recovery from "dry ashing" is related to the use of an open or closed system, presence or absence of ashing aids, technique temperature, and specific sample matrix used. In addition, the size of the ashing vessel, amount of ashing aid used, and the total volume of ashing aid solution are all factors that may affect recovery (24). The dry ashing procedure described here is an open system oxidative fusion technique. The findings of this report indicate that endogenous selenium can be quantitatively recovered from selected oyster and fish tissues. The method is simple and requires little attention from the operator. About 30 samples per day can be accommodated with a single one-shelf muffle furnace. The resulting diluted digestate is readily amenable to conventional hydride generation analysis.

The mention of commercial products does not constitute endorsement.

Acknowledgments

For their valued assistance, I thank 3 colleagues at the Columbia National Fisheries Research Laboratory: Joseph Hunn, for his assistance with the ^{75}Se intracoelomic injections; Donald Kane, for his excellent technical performance in sample preparation and hydride generation analyses; and Steve Hamilton for his aid in fish tissue dissection. I also thank E. E. Pickett of the University of Missouri for his helpful suggestions, and Stephen G. Capar of the Food and Drug Administration for supplying the FDA fish tissue standards.

References

(1) Gorsuch, T. T. (1959) *Analyst* **84**, 135–173
(2) Whetter, P. A., & Ullrey, D. E. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 927–930
(3) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Arlington, VA, secs 25.117-25.125
(4) Fiorino, J. A., Jones, J. W., & Capar, S. G. (1976) *Anal. Chem.* **48**, 120–125
(5) Ihnat, M. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 911–922
(6) Flanjak, J. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 1299–1303
(7) Michie, N. D., Dixon, E. J., & Bunton, N. G. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 48–51
(8) Middleton, G., & Stuckey, R. E. (1954) *Analyst* **79**, 138–142
(9) Gleit, C. D., & Holland, W. D. (1962) *Anal. Chem.* **34**, 1454–1457
(10) Mulford, C. E. (1966) *At. Absorpt. Newsl.* **5**, 135–139
(11) Oelschlager, W. (1965) *Landw. Forsch.* **18**, 79–88
(12) Higgs, D. J., Morris, V. C., & Levander, O. A. (1972) *J. Agric. Food Chem.* **20**, 678–680
(13) Heinrich, M., & Kelsey, F. E. (1954) *Fed. Proc.* **13**, 364
(14) Fourie, H. O., & Peisach, M. (1977) *Analyst* **102**, 193–200
(15) Gorsuch, T. T. (1970) *International Series of Monographs in Analytical Chemistry*. **39**, 152 pp.
(16) *Official Methods of Analysis* (1970) 11th Ed., AOAC, Arlington, VA, secs 38.001-38.002
(17) Hoover, W. L., Melton, J. R., Howard, P. A., & Basset, J. W. (1974) *J. Assoc. Off. Anal. Chem.* **57**, 18–21
(18) Uthe, J. F., Freeman, H. C., Johnston, J. R., & Michalik, P. (1974) *J. Assoc. Off. Anal. Chem.* **57**, 1363–1365
(19) LeBlanc, P. J., & Jackson, A. L. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 383–386
(20) Holak, W. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 650–654
(21) Reamer, D. C., & Veillon, C. (1981) *Anal. Chem.* **53**, 1192–1195
(22) Poole, C. F., Evans, N. J., & Wibberley, D. G. (1977)

J. Chromatogr. **136,** 73-83
(23) Holak, W. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 485-495
(24) Tam, G. K. H., & Lacroix, G. (1979) *J. Environ. Sci. Health* **B14,** 515-524
(25) Tam, G. K. H., & Conacher, H. B. S. (1977) *J. Environ. Sci. Health* **B12,** 213-227
(26) Cappon, C. J., & Smith, J. C. (1981) *Arch. Environ. Contam. Toxicol.* **10,** 305-319
(27) Karin, R. W., Buono, J. A., & Fasching, J. L. (1975) *Anal. Chem.* **47,** 2296-2299
(28) Moody, J. R., & Lindstrom, R. M. (1977) *Anal. Chem.* **49,** 2264-2267
(29) Wang, C. H., & Willis, D. L. (1965) *Radiotracer Methodology in Biological Science,* Prentice-Hall, Inc., Englewood Cliffs, NJ, Chapter 9.
(30) *The Merck Index* (1976) 9th Ed., Merck and Co., Inc., Rahway, NJ
(31) "Selenium" (1976) Committee on Medical and Biological Effects of Environmental Pollutants, National Academy of Sciences, Washington, DC, Chapter 1
(32) Natrella, M. G. *Experimental Statistics* (1966), National Bureau of Standards Handbook 91, U.S. Government Printing Office, Washington, DC, Chapter 1
(33) Fogg, D. M., & Wilkinson, N. T. (1956) *Analyst* **81,** 525-531
(34) Crosby, N. T. (1977) *Analyst* **102,** 225-268
(35) Van Loor, J. C. (1980) *Analytical Atomic Absorption Spectroscopy,* Academic Press, New York, NY, Chapter 4
(36) Bem, E. M. (1981) *Environ. Health Perspect.* **37,** 183-200

Hydride Generation and Condensation Flame Atomic Absorption Spectroscopic Determination of Antimony in Raw Coffee Beans and Processed Coffee

ROY W. KUENNEN,[1] MARK H. HAHN,[1] FRED L. FRICKE, and KAREN A. WOLNIK

Food and Drug Administration, Elemental Analysis Research Center, 1141 Central Pkwy, Cincinnati, OH 45202

A method was developed for determining Sb at nanogram per gram levels in raw coffee beans and processed coffee. The procedure uses either total acid digestion or extraction with 6M HCl followed by hydride generation/condensation with subsequent revolatilization of stibine (SbH_3) and detection by flame atomic absorption spectroscopy. The lowest quantifiable level, based on a 2 g (dry weight) sample, is 2 ng Sb/g. The results of recoveries on spiked samples, precision studies on composited coffee samples, and the analysis of National Bureau of Standards Standard Reference Materials demonstrate the reliability and accuracy of the procedure. Sb concentrations in coffee samples were verified by neutron activation analysis and inductively coupled plasma atomic emission spectroscopy. Advantages of the method compared with the AOAC colorimetric procedure and hydride generation without condensation are discussed.

A shipment of raw coffee beans was accidentally contaminated with potentially toxic antimony trioxide (1). Proper assessment of the extent of this contamination required the analysis of coffee beans and processed coffee for microgram and nanogram per gram levels of Sb. To evaluate the potential impact on human health, a comparison of the Sb content of processed coffee made from contaminated and noncontaminated beans was necessary. It was also necessary to analyze processed coffee made from reconditioned coffee beans, i.e., beans that had been contaminated and then "cleaned" by the manufacturer, to ensure the safety of the product.

The method presented here can be used for the routine determination of Sb in coffee samples at the part per billion level. Detection limits for Sb are improved by several orders of magnitude over the AOAC colorimetric method (2), and accuracy and precision at ultratrace levels are improved compared with the hydride-atomic absorption spectroscopic (AAS) method described by Fiorino et al. (3). Total digestion and analysis of the processed coffee samples result in data pertaining to the possible ingestion of Sb by consumers. The 6M HCl extraction of the coffee beans provides a rapid method for determining surface Sb contamination. This type of rapid, routine analysis is necessary to determine the extent of contamination and the success of efforts by the manufacturer to recondition the contaminated beans.

METHOD

Apparatus

See ref. 4.

(a) *Hydride generator.*—Based on design of Fiorino et al. (3). $NaBH_4$ solution and water rinse from pressurized reagent bottles were added to reaction tube through timer-controlled solenoid valves. Operating parameters: 8 mL $NaBH_4$ in 13 s, 2 mL distilled, deionized water in 5 s.

(b) *Condensation tube.*—A 61 cm section of 1.3 cm id corrugated Teflon tubing (SGA Inc., Bloomfield, NJ) was packed half-full with 1 mm Teflon shavings and cooled with liquid nitrogen.

(c) *Gas flow switching valve.*—Three-way valve (Omnifit, Cedarhurst, NY). Helium carrier flow 700 mL/min.

(d) *Atomic absorption spectrophotometer.*—Model 403 (Perkin-Elmer, Norwalk, CT), or equivalent. Operating parameters: Sb electrodeless discharge lamp 217.6 nm, nitrogen flow 6.1 L/min, hydrogen flow 1.9 L/min, 3-slot burner with quartz flame shield.

(e) *Electronic integrator.*—Model 3380S (Hewlett-Packard, Palo Alto, CA), or equivalent. Slope sensitivity 0.3 mV/min; attenuation LOG.

Reagents

(a) *Distilled, deionized water.*—18 Mohm (Millipore Corp., Bedford, MA).

(b) *Acids.*—Concentrated ACS reagent grade, except perchloric, which was double distilled

[1] University of Cincinnati, Department of Chemistry, Cincinnati, OH 45220.

Received December 3, 1981. Accepted March 15, 1982.

0004-5756/82/6505-1146-04$01.00

70% ACS reagent grade (G.F. Smith Co., Columbus, OH).

(c) *Acid diluting solution.*—100 mL HCl plus 100 mL H_2SO_4, diluted to 1.0 L with distilled, deionized water.

(d) *Sodium borohydride solution.*—4%. Dissolve 40 g $NaBH_4$ (98%, Alpha-Ventron, Danvers, MA) in 1.0 L 10% NaOH. Filter with suction through medium porosity glass filter.

(e) *Sb standard solution.*—10 μg/mL. Dilute aliquot of 1000 μg/mL spectroscopy standard (Spex Industries, Inc., Metuchen, NJ), or equivalent, with acid diluting solution.

(f) *Potassium iodide pre-reductant.*—10%. Dilute 10 g KI to 100 mL with acid diluting solution.

Sample Preparation

Handle samples in clean-air environment where possible. Equipment contacting samples during preparation, digestion, and determination should be nonmetallic and scrupulously cleaned with dilute 30% HNO_3 and rinsed at least 5 times with distilled, deionized water. Wear disposable polyethylene gloves (no talc) throughout procedure.

Analyze processed coffee without further preparation with subsamples chosen at random.

Analyze well mixed coffee bean samples for surface Sb contamination without further preparation.

Prepare homogeneity check sample by finely grinding randomly chosen units of selected lot of processed coffee. Grind subsamples with blender to pass 420 μm polypropylene sieve and mix well.

Total Digestion

Weigh 2.0 g processed coffee or composite into 100 mL Kjeldahl flask containing 30 mL nitric–perchloric–sulfuric acids (4 + 1 + 1) (3). Heat flask on micro-Kjeldahl digestion rack, gradually increasing temperature to maintain boiling until all organic matter is destroyed and fumes of SO_3 evolve. Continue heating until vapor ring of H_2SO_4 reaches neck of flask. Cool and transfer remaining H_2SO_4 (5 mL) with several portions of distilled, deionized water to 50 mL volumetric flask containing 5 mL HCl. Dilute to volume with distilled, deionized water. Use 10.0 mL aliquot of this solution for determination of Sb.

HCl Extraction

Weigh 75.0–100.0 g coffee beans into clean, acid-washed 400 mL glass beaker. Extract with three 50 mL portions of warm (65°C) 6M HCl, stirring 5 min. Decant quantitatively through No. 588 pre-pleated filter paper (Schleicher and Schuell Inc., Keene, NH) into 200 mL volumetric flask. Rinse filter with several small portions of warm 6M HCl and dilute to volume with 6M HCl. Pipet 10.0 mL filtrate through second filter into 100 mL volumetric flask. Rinse filter and dilute to volume with distilled, deionized water. Using microliter pipet, dilute 250 mL of this solution with 10 mL acid diluting solution and determine Sb by following method.

Table 1. Analysis of NBS Standard Reference Materials for Sb

Method	Certified value, μg/g	Found, μg/g
Orchard Leaves		
AOAC (2)	2.9 ± 0.3	2.6, 3.0
Hydride generation (3)	2.9 ± 0.3	3.2, 3.4
Hydride generation/ condensation	2.9 ± 0.3	2.9, 3.0
Spinach		
Hydride generation/ condensation	(0.04)[a]	0.034

[a] NBS provisional value.

Sb Determination

Pipet appropriate aliquot of sample from total digestion or HCl extraction or Sb standard solution into reaction tube, add 500 μL KI solution, mix, and wait 1 min. Place tube on generator and open gas-switching valve to connect reaction tube and condensation tube. Cool condensation tube in liquid nitrogen for 45 s. Begin hydride generation. After reaction is finished (18 s), close valve to reaction tube and establish flow of helium through condensation tube. Remove condensation tube from coolant and immerse in 65°C water bath. After 5 s, begin integrating for 30 s. Sb is quantified by method of standard additions by making microliter additions of Sb standard solution to aliquots of sample.

Optimize flame conditions before analysis with 10 μL Sb standard solution in 10 mL acid diluting solution.

Reanalyze samples with nondetectable levels of Sb, using 2 g sample diluted to 25 mL and one point standard addition.

Results and Discussion

Initial efforts to quantify Sb in processed coffee by the AOAC colorimetric procedure (2) were

Table 2. Recovery of Sb, using hydride generation/condensation AAS method

Added, μg/g[a]	No. of detns	Rec., %, ± SD	Range
0.050	3	90 ± 4	86–94
0.10	15	99 ± 8	84–120
0.20	3	100 ± 4	95–103

[a] Sb content of unspiked processed coffee was <0.002 μg/g.

Table 3. Precision obtained, using total digestion and 6M HCl extraction procedures

Sample	No. of detns	Procedure	Sb, μg/g, ± SD	RSD, %
Processed coffee composite	10	total digestion	0.014 ± 0.004	28
Reconditioned coffee beans	19	6M HCl extraction	1.05 ± 0.11	10

unsuccessful. In that procedure, after digestion of the sample, pentavalent Sb in dilute hydrochloric acid is reacted with Rhodamine B to form a complex that is extracted into an organic solvent and absorbs at 565 nm. This method is reliable at the microgram per gram level as shown in Table 1; however, it is not sufficiently sensitive to enable analysis of processed coffee at the nanogram per gram level.

Although the hydride generation AAS procedure (3) without condensation demonstrated excellent precision and accuracy at Sb levels greater than 100 ng/g, the reaction by-products produced during the generation of stibine caused disturbances in the flame that resulted in a high background signal and therefore deterioration of the detection limit. The atomic absorption spectrometer used in this study was not equipped with background correction. The condensation technique, in which stibine is trapped in a liquid nitrogen-cooled condensation tube while interfering by-products (mainly hydrogen) pass through, permits separation of the stibine from the background, resulting in an order of magnitude improvement in the detection limit. The detection limit is calculated as the concentration equivalent to twice the standard deviation (SD) of the measurement of the blank solution. Blank levels varied from nondetectable (no peak) to 0.2 ng/mL, depending on the reagent lot used.

The condensation/revolatilization process further enhances the sensitivity of the flame AAS

Table 4. Determination of Sb in coffee samples

Sample	Method[a]	No. of detns	Sb, μg/g, ± SD
Initial reconditioned coffee beans	6M HCl extraction HGC-AAS	19	3.6 ± 0.04
Initial reconditioned coffee beans	6M HCl extraction ICP	19	3.6 ± 0.03
Final reconditioned coffee beans	6M HCl extraction HGC-AAS	20	1.05 ± 0.11
Final reconditioned coffee beans	total digestion HGC-AAS	2	1.4, 1.4
All suspect lots processed coffee[b,c]	total digestion HGC-AAS	74	0.013 ± 0.014[d]
Other brands processed coffee	total digestion HGC-AAS	4	0.010, 0.002 <0.002, <0.002
Composite (suspect processed coffee)[b]	total digestion HGC-AAS	10	0.014 ± 0.004
Composite (suspect processed coffee)[b]	NAA	2	0.020, 0.016
Individual subsamples, same lot as composite	total digestion HGC-AAS	4	0.014 ± 0.003

[a] HGC-AAS = hydride generation/condensation atomic absorption spectroscopy, ICP = inductively coupled argon plasma, NAA = neutron activation analysis.
[b] Processed coffee made from contaminated beans.
[c] Fifty-nine subsamples of 11 different lots and 15 duplicate analyses.
[d] Range 0.002–0.080 μg/g.

determination by concentrating the analyte into a narrow plug before it is introduced into the flame. Optimization of the components of the hydride generation/condensation apparatus has been discussed by Hahn et al. (4). Integration of peak area is necessary because of the number of parameters (e.g., cooling time, bath temperature) for which minor variation affects peak shape. The results presented in Table 1 for the analysis of National Bureau of Standards (NBS) Standard Reference Materials show that this method is accurate for both microgram (Orchard Leaves) and nanogram (Spinach) per gram levels of Sb in organic food matrices.

The linear calibration range for the Sb standard is 2–200 ng, equivalent to 0.2–20 ng/mL in a 10 mL sample aliquot. For sample solutions containing higher concentrations of Sb, the analytical range can be extended by using a smaller aliquot of sample and adjusting the total volume in the reaction tube to 10 mL with diluting solution. An effective linear range of 0.2–20 000 ng/mL can be realized without an extra dilution of the sample digest.

Data for recovery of submicrogram amounts of Sb standard added to the processed coffee just before digestion are presented in Table 2.

The results shown in Table 3 of replicate analyses through the entire procedure of a composited processed coffee sample and of the 6M HCl extraction of 19 subsamples of reconditioned coffee beans (contaminated beans after "cleaning") indicate the precision obtainable with the technique at both nanogram and microgram per gram levels.

A summary of the results of sample analyses by the hydride generation/condensation AAS method as well as neutron activation analysis (NAA) or inductively coupled argon plasma (ICP) atomic emission spectroscopy is presented in Table 4. The first attempt by the manufacturer to recondition the contaminated beans was unsuccessful; however, after a second cleaning process, the beans were acceptable. Confirmatory ICP analyses were performed with a plasma run under standard operating conditions using the Sb emission line at 217.6 nm with Sb quantified by the method of standard additions.

Acknowledgment

NAA of the processed coffee samples was performed by William Cunningham and William Strobe, Food and Drug Administration, Washington, DC.

References

(1) Elinder, C. G., & Frieberg, L. (1979) "Antimony," in *The Handbook on the Toxicology of Metals*, L. Fried, G. F. Nordberg, & V. B. Vouk (Eds), North Holland Biomedical Press, New York, NY, pp. 283–292

(2) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **25.001-25.005**

(3) Fiorino, J. A., Jones, J. W., & Capar, S. G. (1976) *Anal. Chem.* **48**, 120–125

(4) Hahn, M. H., Kuennen, R. W., Caruso, J. A., & Fricke, F. L. (1981) *J. Agric. Food Chem.* **29**, 792–796

PLANTS

Evaluation of Agreement Among Routine Methods for Determination of Fluoride in Vegetation: Interlaboratory Collaborative Study

JAY S. JACOBSON, JOHN J. TROIANO, CHRISTINA C. COSENTINI, and JOHN EVANS[1]

Boyce Thompson Institute for Plant Research, Tower Rd, Ithaca, NY 14853

An interlaboratory collaborative study was conducted to evaluate the performance of methods and laboratories for the measurement of fluoride in vegetation. Samples of 8 plant species containing about 5–200 ppm fluoride were distributed to 40 participants who were instructed to use their routine methods of analysis. Methods which had sufficient participants to allow the inclusion of results in statistical analyses were classified into 3 categories: (1) Willard-Winter method (similar to the AOAC official final action method); (2) semiautomated and potentiometric methods (similar to the 2 AOAC official first action methods); and (3) potentiometric analysis with prior ashing, fusion, and/or distillation (not an approved method). There was a significant interaction of methods with samples caused by 3 of the 8 samples. In the other 5 samples, the Willard-Winter and semiautomated methods gave higher mean values for fluoride content than did the 2 potentiometric methods. Despite considerable improvement in speed and simplicity of fluoride analyses during the last decade, agreement between laboratories has not improved because of the variety of methods and techniques in use, the inherent differences between methods, and, apparently, poor laboratory quality control.

In recent years, the number of laboratories measuring the fluoride content of vegetation has increased as the number of sources emitting fluorides into the environment has risen. Existing methods for determining fluorides have been modified and new methods have been developed to respond to the need for speed and simplicity. Consequently, a greater variety of methods are in use. Personnel with varying skill, training, and experience in fluoride analyses are employed, and quality control procedures may not be used in order to save time and expense. For these reasons, there is a continuing need for evaluation of between-laboratory agreement.

Received December 2, 1981. Accepted March 5, 1982.

[1] Cornell University, Biometrics Department, Ithaca, NY 14853.

This report of the Associate Referee, J. S. Jacobson, was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

The major sources of variation in measurements of the fluoride content of vegetation are known from previous studies. The greatest source of variation is due to practices used in sampling field-grown vegetation. Once samples are collected, the greatest source of variation is between laboratories. The magnitude of variation in results is affected by the amount of fluoride in samples and by the type of vegetation sampled (1,2).

AOAC uses collaborative studies to evaluate methods (3), but such studies are also useful for comparisons of between-laboratory agreement (4). This collaborative study was conducted to compare different methods for the determination of fluoride in vegetation and to evaluate the performance of laboratories. No attempt was made to identify and include only laboratories acknowledged as competent in fluoride analyses. The samples included a wide variety of plant species with fluoride contents in the usual range of interest (5–200 ppm). To avoid overburdening analysts and thus diminishing the number of participants, duplicate samples were not distributed nor were duplicate analyses requested. Information about methods and techniques of analysis, procedures for quality control, and training of analysts was obtained from questionnaires.

Materials and Methods

All laboratories known to the authors which perform fluoride analyses on vegetation were invited to join the study. Samples of dried plant material were pulverized to pass a 40 mesh sieve, mixed thoroughly to ensure homogeneity, and distributed in 5 g aliquots to participants. Instructions, standard report forms, and questionnaires concerning procedures used, quality control, and training of analysts were sent with the samples. Samples were coded as follows: A, balsam fir needles; B, citrus leaves; C, loblolly pine needles; D, cherry leaves; E, milo maize leaves; F, pasture grass; G, eucalyptus leaves; and H, hay. Laboratories were identified with code

0004-5756/82/6505-1150-05$01.00

numbers to preserve anonymity, and were instructed to use their usual staff and routine methods, to perform one analysis per sample, and to submit results and completed questionnaires within a certain period of time.

Five laboratories used the Willard–Winter method (I) (similar to AOAC official final action method **25.049–25.055**); 11 used the semiautomated method (II) (similar to AOAC official first action method **3.083–3.095**); 14 used the potentiometric method (III) (similar to AOAC official first action method **3.077–3.082**); and 7 used potentiometric analysis with prior ashing, fusion, and/or distillation (IV). Only 3 laboratories used other methods (oxygen flask combustion with potentiometric analysis (V); acid digestion with semiautomated analysis (VI); and neutron activation analysis (VII)), so these results could not be included in subsequent statistical analyses. For each method, laboratories with abnormally high or low bias were identified by a rank sum test (5). Results were ranked by method in each

Table 1. Collaborative results for determination of fluoride (ppm) in plant samples[a]

	Sample[b]							
Method	A	B	C	D	E	F	G	H
I	64.5	4.4	98.1	88.5	149	40.5	223	68.3
I	68	8	99	91	158	24	207	72
I	60	12	94	76	96	40	217	68
I	75	3.4	120[c]	97	110	47	240	69
I	57.1	11.1	99.1	91.3	146.0	47.0	227.5	73.4
II	67	5	103	88	168	38	262	69
II	56	18[c]	73	125	144	21	218	69
II	77	5.8	105	81	158	42	226	62
II	66	1.7	92	79	147	35	210	67
II	83	8	117	99	160	51	239	78
II	57.6	3.16	98.3	83.7	157.3	35.2	195.4	65.3
II[d]	100.8	18.9	122.2	123.2	143.2	63.7	203.4	98.7
II	83	2	112	96	176	44	229	86
II	67.4	2.89	98.0	85.6	160	41.7	229	71.1
II	49	2	77	69	110	30	190	57
II	74.0	3.8	103.0	93.0	161.0	39.0	199.0	67.0
III[d]	59	2.5	105	86	158	36	173	66
III	61	2.0	93.5	82.8	150.2	37.6	139.9	61.6
III	57	10[c]	90	72[c]	150	31	143	54
III	61	6	94	83	143	42	171	63
III	69.3	1.8	103.2	89.2	167	43.7	201	77.5
III	59	4.1	104	93	144	36	190	74
III[d]	43	1.5	83	67	130	28	126	58
III[e]	64	12	74	75	88	42	136	39
III	62	2	104	90	161	38	210	70
III	61	1.9	102	88	154	40	213	62
III	64	3	104	86	166	42	234	62
III	62	1.8	102	85	174	40	182	64
III	63	2	98	87	150	38	152	65
III	56.2	2.7	94	88.6	127	35.6	137	56.5
IV	59.8	<3.9	79.8	39.3[c]	149.1	34.3	225.7	81.8
IV[d]	77	7.4	100.0	98	150	52	240	78
IV	60	0[c]	88	78	140	35	201	61
IV	67	5.2	99	91	156	43	226	72
IV	59	6	96	80	132	40	167	69
IV	52	5	100	89	155	59[c]	210	114[c]
IV	75	5	96	83	109	38	174	60
V	65.7	—[f]	82.2	73.7	139	9.8	178.3	58.8
VI	62	0	105	85	143	38	210	60
VII	69	<7	<23	79	105	54	141	57

[a] Each row shows the results for a single laboratory performing one determination on each sample by the method shown: I, Willard–Winter; II, semiautomated; III, potentiometric with extraction; IV, potentiometric with ashing, fusion, and/or distillation; V, potentiometric with oxygen flask combustion; VI, semiautomated with acid digestion; VII, neutron activation analysis.

[b] See text for description of samples.

[c] Extreme values, deleted from study.

[d] All values from this laboratory deleted from study; results biased.

[e] All values from this laboratory deleted from study; laboratory variances unusually high.

[f] Sample lost.

Table 2. Split-plot ANOVA of ranked data for 8 vegetation samples

Source of variation	DF	Mean square
Methods	3	782.1535*
Laboratories within methods	27	235.6425
Methods × samples	21	116.8876**
Residual	178	50.7581

* Significant at $P = 0.05$.
** Significant at $P = 0.01$.

sample, and the ranks were summed across samples for each laboratory. Variances of ranks were subjectively compared and those laboratories with high variances were not included in the study. Also, individual outlying values were identified for each method and sample using Dixon's test (5).

The data, with outliers removed, were analyzed in a split-plot analysis of variance (ANOVA) with method as the whole-plot factor and sample as the sub-plot factor. Before the ANOVA, the homogeneity of between-laboratory variances was determined to validate results from the whole- (i) and sub-plot (ii) sections of the ANOVA. The homogeneity of between-laboratory variances was tested for (i) 4 methods averaged over samples and (ii) within methods for the 8 samples by using Bartlett's test (6). A test of (i) did not show significant heterogeneity, whereas a test of (ii) was highly significant ($P = 0.01$) and invalidated the sub-plot analysis. The data then were ranked within each sample, which produced no significant heterogeneity of variance in either (i) or (ii). Therefore, an ANOVA was conducted on the ranked data set. The variation among samples (7 degrees of freedom) was not included in the sub-plot analysis because the ranking of laboratories within each sample constrained their respective rank sums to be equal and the corresponding sums of squares to be zero. For comparisons of methods, pre-specified orthogonal contrasts were made. Both methods III and IV employed the potentiometric method for measuring fluoride so the mean of their rank sums was compared with that of methods I and II. Rank sums also were contrasted between methods I and II and between methods III and IV. The general approach of using ANOVA on data from collaborative studies after screening for outliers and testing for homogeneity of variances has been suggested previously (7).

Results

The fluoride contents of 8 vegetation samples reported by 40 laboratories are presented in Table 1. The rank sum test indicated that results from 4 participants should be deleted from the study, and the comparison of rank variances indicated that results from one additional laboratory also should be deleted. In addition, 8 individual values were identified as outliers (Table 1).

There was a significant difference among methods in the whole-plot section of the ANOVA of the ranked data for the 8 samples. The average values of the rank sums of the Willard-Winter and semiautomated methods (methods I and II) were similar, but different from the average rank sums for the 2 potentiometric methods (methods III and IV) (Tables 2 and 3). In addition, the methods × samples interaction was highly significant ($P = 0.01$) indicating that the order or ranking of methods differed depending on which sample was analyzed. The rank sums for methods were similar in Samples A, D, F, G, and H, but dissimilar in Samples B, C, and E. Thus, a split-plot ANOVA was performed on the 5 similar samples and separate ANOVAs on the other 3 samples. This procedure eliminated the methods × samples interaction because nearly all of the variation due to methods was in the highly significant contrast of the average rank sums for methods I and II compared with that of methods III and IV (Table 4). ANOVA of Samples B, C, and E showed significant differences in ranks between methods I and II for Sample E and between methods III

Table 3. Rank sum of each method in each sample and average across samples

Method	Sample								Av. across samples
	A	B	C	D	E	F	G	H	
I	18.0	21.7	14.1	18.5	10.2	24.5	23.8	20.6	19.1
II	22.9	16.9	20.0	18.6	21.6	17.4	22.5	20.2	20.0
III	13.4	7.6	17.5	14.8	19.0	14.1	10.3	11.8	13.7
IV	13.6	20.5	10.3	11.0	11.0	12.5	15.7	16.2	13.6

Table 4. Split-plot ANOVA of ranked data from Samples A, D, F, G, and H

Source of variation	DF	Mean square
Methods	3	
Contrast of:		
Methods I and II vs III and IV	1	2273.9075**
Method I vs II	1	8.8817
Method III vs IV	1	9.6200
Laboratories within methods	28	193.6604
Samples (S) × methods	12	
Contrast of:		
S × Methods I and II vs III and IV	4	111.0790
S × Method I vs II	4	243.8144
S × Method III vs IV	4	313.8144
Residual	96	46.6489

** Significant at $P = 0.01$.

and IV for Samples B and E. A significant difference in the average ranks of methods I and II compared with that of methods III and IV was found for Sample B. There were no significant differences between methods for Sample C (Table 5).

Discussion

The analysis of data reported in this study revealed that some laboratories obtained biased or imprecise results and that, for some vegetation samples, a significant source of between-laboratory variation can be attributed to the use of different analytical methods.

In a previous study in which results from the Willard-Winter, semiautomated, and potentiometric methods were compared, the potentiometric method gave significant negative bias with 2 of the 7 plant species tested (8). The 2 potentiometric methods employed in the current study also gave lower results than did the Willard-Winter and semiautomated methods in 5 of 8 samples. A major reason for using ashing, fusion, and/or distillation is to overcome any deficiencies of the extraction procedure. Further study is needed to identify the reasons for differences in results between methods and for the influence of different types of vegetation samples on the performance of analytical methods.

Although it is known that fluoride methods are sensitive to procedure modifications, only 8 laboratories in this study followed a published, standardized method (9–11). Many differences in procedures, techniques, and equipment exist, even among laboratories using the same method. To obtain results in close agreement, a group of laboratories should: (*1*) use carefully trained and experienced personnel; (*2*) select one standardized method and adhere rigidly to a single version of that method; (*3*) practice internal quality control by analyzing portions of the same control vegetation sample every day that unknown samples are analyzed; and (*4*) regularly compare results among laboratories (12). These procedures would improve precision, but they do not guarantee that accurate results will be obtained. For this purpose, we suggest that the National Bureau of Standards prepare reference vegetation samples certified for fluoride. Most participants in this study indicated that they would

Table 5. Separate ANOVAs of ranked data from Samples B, C, and E

	Sample B		Sample C		Sample E	
Source of variation	DF	Mean square	DF	Mean square	DF	Mean square
Methods	3		3		3	
Contrast of:						
Methods I and II vs III and IV	1	333.7189**	1	71.5500	1	13.2490
Method I vs II	1	72.0750	1	79.5018	1	396.0333*
Method III vs IV	1	367.3391**	1	250.0245	1	310.7539*
Residual	24	41.0549	27	76.0002	28	71.4506

* Significant at $P = 0.05$.
** Significant at $P = 0.01$.

purchase standard reference samples if they were available in the range of about 5–200 ppm.

Acknowledgments

The study was made possible by contributions of plant material from several of the participants in the study. Larry Heller, John Laurence, and D. C. McCune critically reviewed the manuscript and made valuable suggestions.

References

(1) Jacobson, J. S., & McCune, D. C. (1969) *J. Assoc. Off. Anal. Chem.* **52,** 894–899
(2) Jacobson, J. S., & McCune, D. C., (1972) *J. Assoc. Off. Anal. Chem.* **55,** 992–998
(3) *Handbook of the AOAC* (1977) 4th Ed., AOAC, Arlington, VA
(4) Youden, W. J. (1967) *Statistical Techniques for Collaborative Tests*, AOAC, Arlington, VA
(5) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA
(6) Snedecor, G. W., & Cochran, W. G. (1967) *Statistical Methods*, 6th Ed., Iowa State University Press, Ames, IA, 296 pp.
(7) McArthur, D. S., Baldeschwieler, E. L., White, W. H., & Anderson, J. S. (1954) *Anal. Chem.* **26,** 1012–1018
(8) Jacobson, J. S., & Heller, L. I. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 150–153
(9) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **3.077-3.095, 25.049-25.055**
(10) *Annual Book of ASTM Standards* (1981) American Society for Testing and Materials, Philadelphia, PA, Methods D3269-3271
(11) *Methods of Air Sampling and Analysis* (1977) 2nd Ed., M. Katz (Ed.), American Public Health Association, Washington, DC, pp. 384–421
(12) McCully, K. A., & Lee, J. G. (1980) in *Optimizing Chemical Laboratory Performance through the Application of Quality Assurance Principles*, F. M. Garfield, N. Palmer, & G. Schwartzman (Eds), AOAC, Arlington, VA, pp. 57–86

STERILITY TESTING

Evaluation of Glutaraldehyde and Hydrogen Peroxide for Sanitizing Packaging Materials of Medical Devices in Sterility Testing

SAMUEL ESKENAZI, OLGA E. BYCHKOWSKI, MARILYN SMITH, and JAMES D. MACMILLAN[1]
Food and Drug Administration, 850 Third Ave, Brooklyn, NY 11232

External surfaces of packaging materials used for sterile medical devices may introduce contaminants into working areas used for sterility testing. Light wiping with tissues moistened with alkaline 2% glutaraldehyde (Cidex) or 3% hydrogen peroxide effectively reduced counts on 5 × 8 cm strips of packaging material (Tyvek) inoculated with 10^7 spores of *Bacillus subtilis*. The ability of antimicrobial agents to penetrate packaging material and kill contaminants on the medical device was tested by inoculating filter membranes with ca 100 cells of *Pseudomonas aeruginosa* or *Staphylococcus aureus* or ca 100 spores of *Bacillus subtilis*. A sterile square of test packaging material placed over the inoculated membrane (direct method) or 0.5 cm above the membrane (indirect method) was wiped with the antimicrobial agent. Except for polyethylene film (3 mil), all materials tested, including glassine and several types of coated and uncoated Tyvek, were penetrated by the agents, killing cells on the inoculated membranes. Death rates varied, depending on the organism, packaging material, and testing method. It is suggested that penetration tests be performed before using antimicrobial agents for sanitizing packaging materials during sterility tests.

Medical devices must be tested to determine whether they are free of viable microorganisms. Details of the procedure used for sterility testing are found in the current *United States Pharmacopeia* (USP) (1). Detection of contaminating organisms is based on growth in culture media; therefore, a low contamination level must be maintained in the immediate testing area. Any growth detected in culture media must come from the product itself, not from the environment or external packaging.

Although most medical products are adequately sterilized, the external surface of the package usually becomes contaminated during storage, shipping, or handling. Viable microorganisms on the surface or embedded in the fibers of the packaging materials could contaminate the media or equipment used in the sterility test and give a false-positive result. To minimize this possibility, most sterility tests are conducted in a laminar flow hood or in a bio-clean room. The USP sterility test procedure for drugs requires that the exterior surfaces of ampules and the closures of vials and bottles be cleansed with a suitable decontaminating agent. A sanitation procedure is not outlined for medical devices; however, aseptic techniques are required for opening packages. Surface contamination can be reduced by lightly wiping the package with a sterile cloth or tissue dampened in an antimicrobial agent.

Materials used for packaging medical devices include glassine, polyolefin (Tyvek), surgical papers, plastic films, metal foils, and coated or laminated combinations such as coated Tyvek and plastic film bonded to metal foil. These materials vary in their ability to act as barriers to penetration by microorganisms, moisture, oxygen, and light. Several methods have been developed to test for bacterial penetration (2–10).

Studies have shown that 2% glutaraldehyde and 3% hydrogen peroxide are effective antimicrobial agents, active against sporeforming (11–21) and nonsporeforming bacteria (15, 16, 18–25), fungi (12–16, 26), and viruses (13–15, 18, 27, 28). The effectiveness of these agents in reducing surface contamination of packaged medical devices and their ability to penetrate the packaging material were investigated. Penetration by these agents could destroy potential contaminating microorganisms and thus produce false-negative results in sterility tests conducted on the packaged medical device.

METHOD

Antimicrobial Agents

(a) *Cidex.*—2% aqueous solution of glutaraldehyde (Arbrook, Inc., Arlington, TX 76010). Solutions of alkaline glutaraldehyde (pH 7.5–8.5)

Received December 4, 1981. Accepted February 12, 1982.
Presented in part at the 93rd Annual Meeting of the AOAC, Oct. 15–18, 1979, at Washington, DC.

[1] Department of Biochemistry and Microbiology, New Jersey Agricultural Experiment Station, Cook College, Rutgers University, New Brunswick, NJ 08906.

0004-5756/82/6505-1155–07$01.00

were prepared fresh daily by addition of buffer salts provided by the manufacturer.

(b) *Hydrogen peroxide.*—3%, laboratory grade (Fisher Scientific Co., Fairlawn, NJ 07416).

Media and Reagents

(a) *Trypticase soy broth and trypticase soy agar.*—(BBL, Cockeysville, MD 21030).

(b) *Dehydrated letheen agar.*—Medium containing lecithin and Tween 80 to neutralize effects of some antimicrobial agents (Difco Laboratories, Detroit, MI 48232).

(c) *Glycine.*—(Fisher Scientific Co.) A 2% aqueous solution was used to neutralize the antimicrobial effects of glutaraldehyde.

Cultures

Staphylococcus aureus (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), and *Bacillus subtilis* (ATCC 6633) were purchased from American Type Culture Collection (ATCC), Rockville, MD 20852. For some studies on hydrogen peroxide, cultures of *B. subtilis* (ATCC 6633) and *P. aeruginosa* (ATCC 27853) were obtained from Difco Laboratories. Cultures were maintained on trypticase soy broth and transferred weekly.

Nonsporeforming bacteria were grown overnight at 35°C in trypticase soy broth. Before use, cultures were diluted with fresh sterile broth to absorbance 0.5 (660 nm) to give cell concentration of ca 3×10^8/mL. Further dilutions were made in broth to obtain counts of 100–200 organisms/mL.

B. subtilis was grown for 5 days at 35°C on trypticase soy agar. Cells were harvested and suspended in 95% ethanol to kill vegetative cells. Spores were washed 3 times in distilled water, collected by centrifugation, and resuspended in distilled water. Spore preparations were stored at 4°C and viable spores were counted weekly by plating on trypticase soy agar. Dilutions of spores (either 10^7 or 100–200/mL) were prepared on the basis of these counts and confirmed by plating.

Packaging Materials

Packaging materials included polyethylene film, 3 mil (Tower Products, Mundelein, IL 60060); Tyvek® type 10 spunbonded olefin, style 1073B (E.I. DuPont de Nemours & Co., Wilmington, DE 19898); Ascot type 101 (Tyvek 1073B coated with heat-sealable adhesive) (Appleton Papers Inc., Appleton, WI 54912); Perfecseal SBP-3A, Tyvek styles 1059B and 1073B, both coated with thermoplastic adhesive (Paper Manufacturers Co., Southhampton, PA 18966); Glassine syringe envlopes, Chieftain® (American Hospital Supply Co., Evanston, IL 60204).

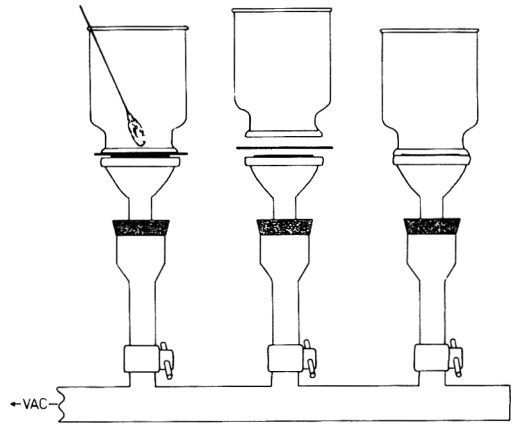


Figure 1. Three stages of direct contact procedure for testing penetration of packaging material by antimicrobial agents. Filter funnel (right) contains membrane filter inoculated with test bacteria. Funnel is raised (middle) and sample of packaging material is inserted over inoculated membrane. Funnel is replaced (left) and packaging material is swabbed with antimicrobial agent.

Effectiveness of Antimicrobial Agents

Samples of Tyvek 1073B were cut into 5 × 8 cm strips, sterilized with ethylene oxide, and inoculated with ca 10^7 spores of *B. subtilis*. After drying overnight at room temperature, inoculated surfaces were wiped lightly with sterile laboratory tissues dampened with 2% glutaraldehyde or 3% hydrogen peroxide. Ten strips were prepared at the beginning of each experiment. The wiped strips were air dried on a sterile surface; at timed intervals a strip was placed in a blender with 100 mL peptone water containing 0.1% Tween 80 (hydrogen peroxide experiments) or 2% glycine (glutaraldehyde experiments). Strips were blended for 1 min to suspend spores; viable counts were determined by plating the liquid on letheen agar incubated for 48 h at 35°C.

Penetration of Packaging Materials by Antimicrobial Agents

Two procedures were employed. In the first, inoculated membrane filters served as simulated product in direct contact with packaging material. Suspensions containing 100–200 cells were placed on 0.45 μm membrane filters in a manifold set-up with 6 funnels (Figure 1). Each membrane received the same number of cells. The liquid was removed by vacuum filtration

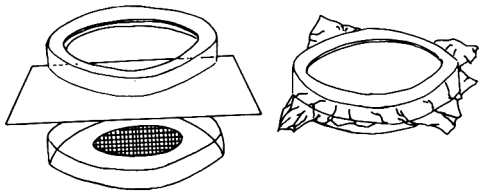


Figure 2. Indirect contact procedure for testing penetration of packaging material by antimicrobial agents. Inoculated membrane filter is placed on agar surface in petri dish. Square of packaging material is placed under modified petri dish cover. Large hole in center of cover permits wiping material with disinfectant.

and membranes were air dried in place for 2 h. The upper portion of the funnel was raised and a square piece (6.5 × 6.5 cm) of test packaging material was inserted over the inoculated membrane. A sterile cotton swab was moistened with antimicrobial agent, and excess fluid was expressed. To simulate the wiping procedure of a packaged product, 5 test strips were lightly swabbed. Membranes were removed individually at various intervals and placed on plates of letheen agar. After incubation for 48 h at 35°C, colonies were counted and compared with those plated with an unswabbed control.

The second procedure (indirect contact) was used to determine whether vapors containing disinfectant could penetrate packaging material and kill cells. Membrane filters in a manifold were inoculated and liquid was removed as described. Membranes were dried for 2 h and placed individually on the surface of letheen agar in 15 × 100 mm petri dish bottoms. A piece of packaging material ca 10 × 10 cm sq. was placed over the petri dish bottom so that it rested on the upper rim. The packaging material was held in place ca 0.5 cm above the membrane with a petri dish top from which the center had been removed to provide an opening ca 7 cm in diameter (Figure 2). Through this opening, the packaging material was swabbed with disinfectant. Six membranes were prepared for each experiment; at various intervals, the packaging material was removed from one of the plates and the modified petri cover was replaced with an intact one. Inoculated membranes were thus exposed to any vapors which might have penetrated the packaging materials. After 48 h at 35°C, colonies were counted and compared to those plated with an unswabbed control.

Results and Discussion

Figure 3 shows the effectiveness of glutaraldehyde in reducing the viable count on squares

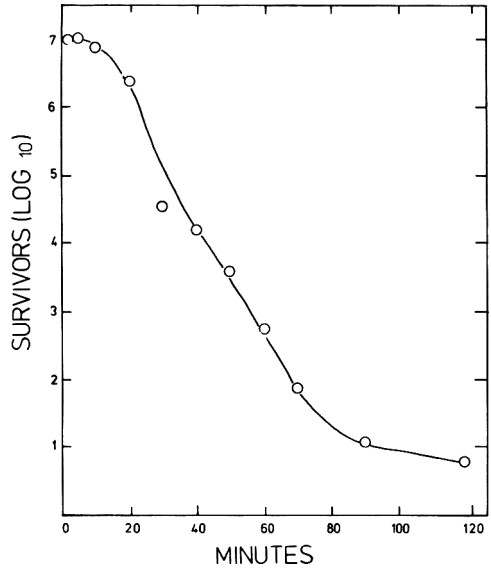


Figure 3. Effectiveness of 2% glutaraldehyde in reducing viable count on uncoated Tyvek 1073B inoculated with *B. subtilis* spores.

of Tyvek 1073B (uncoated) inoculated with about 10^7 spores of *B. subtilis*. After the surface was wiped with the antimicrobial agent, there was a 10 min lag before the counts began to decrease. The time required to reduce the population by one log cycle (D-value) was about 12 min during the rapid kill-off period that occurred between 10 and 80 min. This result agrees well with that of Sykes (29) who reported that 10^6 *B. subtilis* spores were killed in 1 h (D = 10 min) when spores were added directly to 2% glutaraldehyde. We did not test nonsporeformers, which have been shown to be considerably more sensitive than spores. Similar experiments were repeated with a number of other packaging materials inoculated with *B. subtilis* spores, and it became clear that wiping with glutaraldehyde was effective in sanitizing the surface.

The next series of experiments were designed to determine whether glutaraldehyde could penetrate packaging materials and kill potential internal contaminants. Figure 4 shows a typical result obtained with Tyvek 1073B (uncoated) using the direct contact method. Viable counts of *P. aeruginosa* and *S. aureus* decreased from >100 cells to 20 and 5 cells, respectively, within 10 min. Counts of inoculated squares not exposed to glutaraldehyde did not decrease during this time. Enough glutaraldehyde penetrated the uncoated Tyvek to affect viability of *B. subtilis*

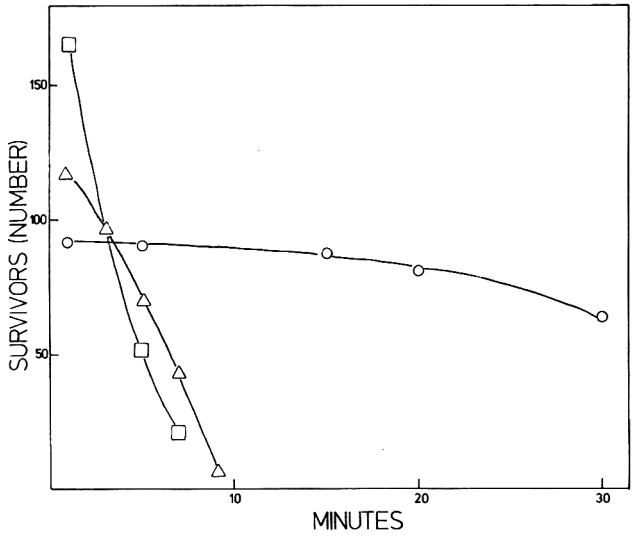


Figure 4. Ability of 2% glutaraldehyde to penetrate uncoated Tyvek 1073B and kill *B. subtilis* (○), *S. aureus* (△) and *P. aeruginosa* (□). Direct contact method was used (see Figure 1).

spores, and by 30 min there were only about 70% survivors. As before, inoculated controls did not show a decrease.

Even potential contaminants about 0.5 cm from the uncoated Tyvek packaging material were susceptible to death after the external surface was swabbed with glutaraldehyde (indirect contact method). Counts of *S. aureus* decreased from 131 to 0 in 7 min after swabbing (Figure 5). Polyethylene film, however, was considerably more resistant to penetration. In the example shown in Figure 5, no death of *S. aureus* occurred within at least 45 min. The lower count observed at 60 min was not observed in subsequent experiments with plastic film.

Results of penetration tests on the other packaging materials by the two methods are summarized in Table 1. Survivor curves were plotted and the time required for 50% reduction in cell numbers was estimated. Although not all test organisms were used in each case, some general observations can be made. All materials

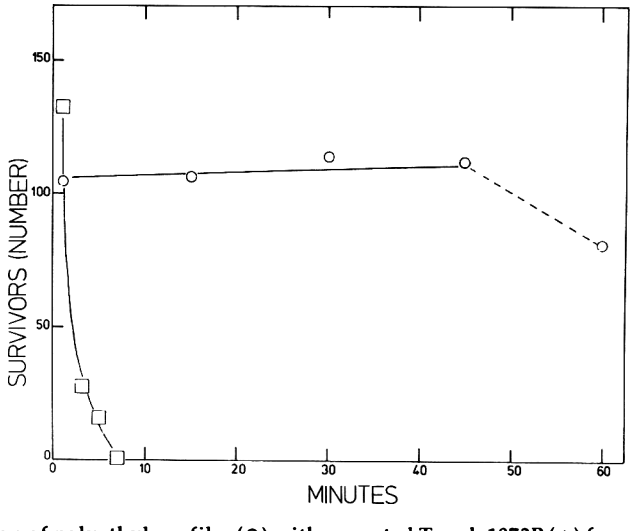


Figure 5. Comparison of polyethylene film (○) with uncoated Tyvek 1073B (△) for penetration by 2% glutaraldehyde and killing of *S. aureus*. Indirect contact method was used (see Figure 2).

Table 1. Time (min) required to reduce viable counts on inoculated membrane filters by 50% after swabbing packaging material with glutaraldehyde

Packaging material	Direct contact[a]			Indirect contact[a]		
	BS	PA	SA	BS	PA	SA
Ascot 101 (coated Tyvek 1073B)		16	21	ND(15)[b]	ND(15)[b]	ND(15)[b]
Glassine envelopes	33[c]	0.5	1.4		10[c]	11[c]
Polyethylene film		ND(60)[b]	ND(60)[b]		ND(60)[b]	ND(60)[b]
Tyvek 1073B (uncoated)	33	2.9[d]	3.5[e]		4.7[d]	3.1[c]
Tyvek SBP-3A/1073B (coated)	ND(120)[b]	1.0	8.0			14.5
Tyvek SBP-3A/1059B (coated)	ND(120)[b]	2.5	15.5	ND(60)[b]	11.0	7.5[c]

[a] BS = *Bacillus subtilis*, PA = *Pseudomonas aeruginosa*, SA = *Staphylococcus aureus*.
[b] ND = no death detected; experimental time (min) in parentheses.
[c] Average of 2 determinations.
[d] Average of 4 determinations.
[e] Average of 3 determinations.

tested except polyethylene film were penetrated by the glutaraldehyde. With the direct contact method, glassine appeared to be the most penetrable material, followed by uncoated Tyvek and the 3 varieties of coated Tyvek. Tyvek is water-resistant but not waterproof; it is penetrable by aqueous solutions of glutaraldehyde or by glutaraldehyde vapors. The coated varieties are more resistant, and the type and thickness of the coating apparently affects the amount of penetration. Although both Ascot 101 and coated Tyvek SBP-3A/1073B are made from the same grade and style of Tyvek, the adhesive coatings are applied by different manufacturers. The time required to kill *P. aeruginosa* and *S. aureus* by the direct contact method differed widely with the 2 products, and Ascot 101 seemed less permeable.

Except in tests of Ascot 101 and polyethylene film, death of vegetative cells was also observed with the indirect contact method. Evidently, the amount of glutaraldehyde vapor accumulating within the petri dish was sufficient to kill organisms about 0.5 cm away from the packaging material.

As expected, cell death was usually more rapid with the direct contact method. However, in 2 cases (uncoated Tyvek 1073B and coated Tyvek SBP-3A/1059B) 50% kill of *S. aureus* occurred in less time with the indirect method. In the former material this could be an anomalous result since 3.1 min was the average of 2 widely varying determinations, 5.8 and 0.5 min.

With the direct contact method, 3 test organisms gave consistent results in susceptibility to death. Spores were considerably more resistant to glutaraldehyde than were the nonspore-forming bacteria, and *S. aureus* always took longer to kill than did *P. aeruginosa*. Although fewer data were collected by the indirect contact method, in 2 cases (uncoated Tyvek 1073B and coated Tyvek SBP-3A/1059B) *S. aureus* appeared to be the more sensitive organism. Once again, this was the average of widely diverse values obtained in separate experiments.

Glutaraldehyde penetrated all of the materials tested except polyethylene film. To determine whether another antimicrobial agent would give similar results, we tested hydrogen peroxide (3%), which is a known sporicidal agent with a mode of action different from that of glutaraldehyde.

In a preliminary experiment, the effectiveness of 3% hydrogen peroxide in reducing viable counts of 10^6 *B. subtilis* spores inoculated on squares of Tyvek 1073B (coated) was determined. The D-value was about 20 min.

With *B. subtilis* and *P. aeruginosa* as test organisms, 4 types of packaging material were analyzed for penetration by hydrogen peroxide (Table 2). All materials tested except polyethylene film were penetrated. As expected, coated Tyvek was less permeable than uncoated Tyvek. Surprisingly, with most cells the vapors (indirect contact method) exhibited a stronger bactericidal action than did direct contact with solutions of hydrogen peroxide. The data suggest that the lethal effects of hydrogen peroxide are more pronounced under the aerobic conditions of the indirect contact method than under the reduced oxygen concentrations possible during the direct contact method. An additional experiment supported this possibility.

About 500 mL of 3% hydrogen peroxide was

Table 2. Time (min) required to reduce viable counts on inoculated membrane filters by 50% after swabbing packaging material with hydrogen peroxide

Packaging material	Direct contact[a]		Indirect contact[a]	
	BS	PA	BS	PA
Tyvek 1073B (uncoated)	20	1.1	4.3	2.4
Tyvek SBP-3A/1073B (coated)	D[b]	8.3	5.5	6.3
Polyethylene film	ND[c]	ND	ND	ND
Glassine envelopes	RD[d]	RD	RD	RD

[a] BS = *Bacillus subtilis*, PA = *Pseudomonas aeruginosa*.
[b] D = Death occurred, but only 41% (average of 2 determinations) died during 48 min contact.
[c] ND = No death detected within 2 h after swabbing.
[d] RD = Rapid death observed; no viable counts obtained on first membrane tested immediately after swabbing with hydrogen peroxide.

placed in a flask and air was bubbled slowly through the solution for about 2 h. Squares of uncoated Tyvek placed over inoculated membrane filters were swabbed as in the direct contact method. For aerated and nonaerated hydrogen peroxide, the population was reduced by 50% in 1.6 min and 4.8 min, respectively.

Hydrogen peroxide killed cells very shortly after it was swabbed on glassine, which was the most penetrable material in both experiments. However, it is difficult to understand why cells (including spores) died considerably faster with hydrogen peroxide than with glutaraldehyde (Table 1). A possible explanation is the use of letheen agar as the growth medium. The proteinaceous materials (beef extract and tryptone) in letheen may have inactivated residual glutaraldehyde on the membranes. It is known that amino acids such as glycine and cystine are useful for inactivating glutaraldehyde (30, 31). Assuming that hydrogen peroxide is not inactivated by letheen, the differences observed with the 2 disinfectants could possibly be due to continued action of any residual hydrogen peroxide on the membrane.

Conclusions

Both glutaraldehyde and hydrogen peroxide effectively reduced surface contamination on packaging material. However, they, and perhaps other antimicrobial agents, should not be used to sanitize most packaged medical devices before sterility testing because they can penetrate the packaging material and kill contaminating organisms within. This may not be a problem with devices packaged with impermeable material such as polyethylene film. However, before a disinfectant is used, it is recommended that tests be performed to determine whether penetration is a problem. Alternative methods for sanitizing the surfaces should be investigated. One possibility is the use of high intensity UV light (32). Vigorous wiping with a sterile tissue followed by exposure to a large volume of sterile moving air (e.g., HEPA filter) to dislodge and remove particulates from the surface may reduce the possibility of contaminating the test medium.

Acknowledgments

The authors thank the following, both of FDA New York Regional laboratory: Richard Klein, for invaluable assistance in the preparation of the figures, and Richard F. Meyer, for suggestions on the hydrogen peroxide experiments.

References

(1) *U.S. Pharmacopeia* (1980) 20th Rev., U.S. Pharmacopeial Convention, Inc., Rockville, MD, pp. 879–881
(2) Schneider, P. M. (1980) *Med. Device Diagn. Ind.* 2(5), 28–37
(3) Firpi, M., Carlini, L., & Grillo, S. (1976) *A Study On Microbial Permeability of Paper for Food Packaging, XVI Conference/Alpes Congres*, Grenoble, France, pp. 1–14
(4) Seaman, R. S., Weimar, R. D., Jr, & Maciag, W. J. (1975) *Nonwoven-Product Technology*, INDA Technical Symposium, Miami Beach, FL, pp. 197–207
(5) Adam, W., & Marcy, G. (1977) *Report from the Medical Research Department of the Institute of Hygiene*, Hamburg, West Germany, pp. 1–9
(6) Weeks, M. E. (1974) *The Ability of Steri-Lok Breathable Peel Open Packages to Resist Microbial Penetration Under Use and Abuse Conditions*, 3M Co., St. Paul, MN 55101, pp. 1–7
(7) *German Norms* (1977) *Government Document DIN 58930-Part 2*, Working Committee of the Supply of Sterile Goods of the Medical Norm Committee, DIN German Institute for Norms, Berlin-Tiergarten, West Germany, pp. 1–7
(8) Standard, P. G., Mackel, D. C., & Mallison, G. F. (1971) *Appl. Microbiol.* **22**, 432–437
(9) Standard, P. G., Mallison, G. F., & Mackel, D. C.

(1973) *Appl. Microbiol.* **26,** 59-62
(10) HIMA Report No. 78-4.11 (1978) *Microbiological Methods for Assessment of Package Integrity*, Health Industry Manufacturers Association, Washington, DC
(11) Pepper, R. E., & Chandler, V. L. (1963) *Appl. Microbiol.* **11,** 384-388
(12) Stonehill, A. A., Krop, S., & Borick, P. M. (1963) *Am. J. Hosp. Pharm.* **20,** 465-485
(13) Borick, P. M., Dondershine, F. H., & Chandler, V. L. (1964) *J. Pharm. Sci.* **53,** 1273-1275
(14) Borick, P. M. (1965) *Biotechnol. Bioeng.* **7,** 435-443
(15) Borick, P. M. (1968) *Adv. Appl. Microbiol.* **10,** 291-312
(16) Rubbo, S. D., Gardner, J. F., & Webb, R. L. (1967) *J. Appl. Bacteriol.* **30,** 78-87
(17) Thomas, S., & Russell, A. D. (1974) *J. Appl. Bacteriol.* **37,** 83-92
(18) Snyder, R. W., & Cheatle, E. L. (1965) *Am. J. Hosp. Pharm.* **22,** 321-327
(19) Christensen, R. P. (1977) *J. Dent. Res.* **56,** 822-826
(20) Toledo, R. T., Escher, F. E., & Ayres, J. C. (1973) *Appl. Microbiol.* **26,** 592-597
(21) Spaulding, E. H., Cundy, K. R., & Turner, F. J. (1977) "Chemical Disinfection of Medical and Surgical Materials" in *Disinfection, Sterilization and Preservation*, Block, S. S. (Ed.), pp. 677-684
(22) Ross, P. W. (1966) *J. Clin. Pathol.* **19,** 318-320
(23) Rittenbury, M. S., & Hench, M. E. (1965) *Ann. Surg.* **161,** 127-130
(24) Lane, V., McKeever, J. D., & Fallon, M., (1966) *J. Ir. Med. Assoc.* **58,** 131-132
(25) Schaeffer, A. J., Jones, J. M., & Amundsen, S. K. (1980) *Appl. Environ. Microbiol.* **40,** 337-340
(26) Gorman, S. P., & Scott, E. M. (1976) *J. Pharm. Pharmacol.* **28**(Suppl.), 48
(27) Cunliffe, H. R., Blackwell, J. H., & Walker, J. S. (1979) *Appl. Environ. Microbiol.* **37,** 1044-1046
(28) Saitanu, K., & Lund, E. (1975) *Appl. Microbiol.* **29,** 571-574
(29) Sykes, G. (1970) *J. Appl. Bacteriol.* **33,** 147-156
(30) Gorman, S. P., & Scott, E. M. (1976) *Microbiol. Lett.* **1,** 197-204
(31) Russell, A. D., Ahonkhai, I., & Rogers, D. T. (1979) *J. Appl. Bacteriol.* 46, 207-245
(32) Cerny, G. (1977) *Verpack. Rundsch.* **28,** 77-82

FOOD ADDITIVES

Confirmatory Method for *N*-Nitrosodimethylamine and *N*-Nitrosopyrrolidine in Food by Multiple Ion Analysis with Gas Chromatography-Low Resolution Mass Spectrometry Before and After Ultraviolet Photolysis

W. I. KIMOTO and WALTER FIDDLER
U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 E Mermaid Lane, Philadelphia, PA 19118

A confirmatory procedure is described for determining *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR) in bacon, beer, and malt by gas chromatography-low resolution quadrupole mass spectrometry (GC-MS). The presence of 3 monitored ions, m/z 30, 42, and 74 for NDMA, and m/z 30, 42, and 100 for NPYR, before and disappearance after photolysis under UV light at 365 nm without quantitation of peak areas was considered confirmatory evidence for their presence. The extracts, obtained by mineral oil distillation methods for bacon and malt, and dry column methods for bacon and beer, underwent cleanup procedures before capillary GC-MS analysis. Less than 100 ng nitrosamine in the total extract, or approximately 2 ng NDMA or 3 ng NPYR injected into the GC-MS instrument, can be confirmed by this method. This technique should also be applicable for other volatile nitrosamines.

When detected, volatile nitrosamines are generally present in trace amounts with potentially interfering substances which may lead to false positive results. Most nitrosamines have been shown to be carcinogenic toward many laboratory animal species (1). It is generally accepted that the presence of nitrosamines should be confirmed by mass spectrometry (MS). Many methods have been reported and these have been reviewed in detail (2, 3). While confirmation by gas chromatography (GC)-low resolution MS to yield a complete spectrum is the method of choice, this is not always possible. Most researchers using GC-high resolution MS consider observation of the molecular ion at the same retention time as the standard nitrosamine as the criterion for confirmation (2, 3). The disadvantage of high resolution MS is the cost, which may limit its availability for the routine confirmation of nitrosamines in foods analyzed by government, testing, industry, and university laboratories.

Received December 8, 1981. Accepted March 1, 1982.

GC-low resolution MS systems are more widely available than the high resolution instrument, but they require samples that are much cleaner. Guidelines for a hierarchy of MS confirmatory methods for animal drug residues have been reported (4) and these should be equally applicable to nitrosamines. The first choice is a full-scan spectrum, the second choice is a limited mass scan of a portion of the spectrum that contains only the characteristic information, and the final choice is the monitoring of 3 or more ions. For nitrosamines, the characteristic information is obtained from m/z 30, the ion for NO^+, to the parent ion, so that this will represent a limited scan mass spectrum. For any confirmatory method, the unknown and nitrosamine standard must have the same GC retention time.

Several reports have appeared in which a full-scan mass spectrum was obtained and reported for *N*-nitrosodimethylamine (NDMA) as a confirmatory method in processed fish (5), fish meal (6), malt (7), beer (7, 8), for NDMA and *N*-nitrosopyrrolidine (NPYR) in fried bacon and a beef-like product (9), and for NPYR in fried bacon (8). For multiple ion monitoring, the closer the ratios of the areas of the ions to be monitored are to that of the standard, the more reliable the confirmatory method will be. This method requires less sample than the full-scan mass spectrum method, but the sample must be free of co-eluting contaminants containing one or more of the ions monitored, or any contaminant containing one or more of the monitored ions that will interfere with integration of the peak areas. We believe that this difficulty can be overcome by taking advantage of the fact that nitrosamines are photolabile (10, 11), i.e., by using a procedure similar in principle to a photolytic method we reported as an additional aid to the confirmation of nitrosamines in which GC-thermal energy analyzer (GC-TEA) detection was employed (12). The samples are ana-

0004-5756/82/6505-1162-06$01.00

lyzed quantitatively for nitrosamines by GC-TEA, and then 3 principal characteristic ions of a nitrosamine are monitored by GC-MS before and after photolysis by UV light at 365 nm. We used this technique to confirm the presence of 3 volatile nitrosamines in a few samples in an earlier report (13). The general applicability and details of this technique for the determination of NPYR in fried bacon, and NDMA in malt and beer are reported here.

Experimental

Reagents

(a) *Solvents.*—Dichloromethane (DCM), hexane (dried over anhydrous Na_2SO_4), and pentane, all glass-distilled (Burdick & Jackson Laboratories, Muskegon, MI 49442).

(b) *Sodium sulfate.*—Anhydrous, granular, reagent grade (Mallinckrodt, Inc., St. Louis, MO 63147).

(c) *Silicic acid.*—100 mesh. Activate 4–5 h at 110°C, cool to room temperature, and deactivate with 5 g water/95 g silicic acid (Mallinckrodt, Inc.).

(d) *Alumina.*—Neutral, 100–200 mesh. Activate 3 h at 190°C, cool to room temperature, and deactivate to activity 3 with 6 g water/94 g alumina (Bio-Rad Laboratories, Rockville Center, NY 11571).

(e) *Boiling chips.*—Carborundum, small size (or equivalent).

(f) *N-Nitrosodimethylamine (NDMA).*—Working standard, 0.5 ng/μL DCM.

(g) *N-Nitrosopyrrolidine (NPYR).*—Working standard, 5.0 ng/μL DCM. Caution: NDMA and NPYR are potent animal carcinogens and must be handled appropriately.

Apparatus

(a) *Chromatographic columns.*—Condenser, 200 mm, with 19/38 joints top and bottom (A. H. Thomas Co., Philadelphia, PA 19105, Cat. No. 3215-M20). By glassblowing, lower joint was removed and replaced with stopcock. Also glass column, 15 mm id × 300 mm long with stopcock.

(b) *Evaporative concentrator.*—Kuderna-Danish, 250 mL with 4 mL concentrator tube and Snyder and micro-Snyder distilling columns (Kontes Glass, Vineland, NJ 08360, Cat. No. K-503000-0121, K-570001-0250, K-570050-425, K-569251-0319, or K-569001-0119).

(c) *L-Shaped tube and freezing trap.*—12/5 ball and socket joints with connecting tubing (A. H. Thomas Co., Cat. Nos. 5715-E46 and 5715-J46) were bent in an L-shape. By glassblowing, 19/38 male joint was connected to open end of ball joint, and open end of socket joint was connected to inlet tube of one-piece freezing trap, 195 mm long × 23 mm od.

(d) *Glass wool.*—Pyrex or equivalent.

(e) *Capillary tubes.*—Kimax 1.6–1.8 mm od × 45–50 mm.

(f) *Gas chromatograph–thermal energy analyzer (GC-TEA).*—Previously described (14).

(g) *Gas chromatography–mass spectrometry.*—Hewlett-Packard Model 5992B low resolution quadrupole GC-MS interfaced to HP-9825A desktop computer, HP-9866B printer, and HP-9885M flexible disk drive, and fitted with capillary interface system. GC instrument conditions: 30 m × 0.5 mm id glass capillary column coated with UCON 5100, or 25 m × 0.5 mm id glass capillary column coated with Carbowax 20 M; helium carrier gas 3.5 mL/min; injector 150°C; column 20°C for 2 min, programmed at 8°/min to 160°C, and held at this temperature. For NDMA, 3 ions, m/z 74, 42, and 30, and for NPYR, m/z 100, 42, and 30, were monitored under the following conditions: electron energy 70 eV; electron multiplier 2000–2600 eV; integration time 50 ms/mass monitored. When MS isolation valve was opened, 0.75 mL/min of He flow entered MS instrument and remainder was vented into overhead hood. Data acquired by GC-MS could be processed 1 ion individually or 3 ions simultaneously. More detail could be obtained from the former than from the latter; therefore, the data were processed 1 ion individually. Abundance scale for each ion differed from that of other ions.

(h) *UV source.*—Chromato-Vue Model C-5 (Ultra-Violet Products, Inc., San Gabriel, CA 91778). Medium intensity lamp with 284 microwatts/sq. cm of 365 nm radiation at distance of 457 mm.

Samples and Procedure

(a) Fried bacon samples containing various levels of NPYR were obtained from USDA, Food Safety and Inspection Service monitoring program of commercial producers. Nitrosamines were isolated by a reported modification (14) of a mineral oil distillation procedure (15) or a dry column procedure (16) and cleaned up by the following procedure: Add 15 mL hexane to water-cooled column and place glass wool plug in bottom. Add 5 g alumina and tap column to pack. (Cooling prevents vaporization of pentane and DCM on alumina column.) Top column with 1–2 cm Na_2SO_4. Drain most of hexane

and add 20 mL hexane to column. Add bacon extract, using 5 mL hexane to wash concentrator tube, and add wash to column. Technique of transfer should avoid contact with ground glass joint. Adjust stopcock to maintain flow rate of 1–2 mL/min, and then elute with 50 mL each of 1 + 10, 3 + 10, and 6 + 10 DCM–pentane and collect final fraction in Kuderna-Danish flask. Add boiling chip, fit flask with Synder column, and concentrate solvent on steam bath to 2–4 mL. Replace Synder column with micro-Synder column; then add boiling chip and concentrate solvent to 1.0 mL in 60°C water bath. (Recovery of NPYR varied from 77 to 100%.) With spatula, raise boiling chips above liquid level in tube, wash spatula and boiling chips with a few drops of DCM, and discard boiling chips. Cool extract to 10°C or lower (to prevent bumping) and connect tube to one end of L-shaped tubing containing 19/38 joint; connect other end of tubing, containing ball joint, to freezing trap which is cooled in dry ice–methanol bath and connected to laboratory vacuum system (50–90 mm Hg). Concentrate to 20–40 μL at ambient temperature. Transfer concentrate to 2 capillary tubes sealed at 1 end, and seal tubes. Place 1 tube 2–4 cm from UV light source at 365 nm for 3 h.

(b) Beer malt containing various levels of NDMA was obtained from the Food and Drug Administration. NDMA was isolated by a mineral oil distillation procedure (7), and cleaned up by the following procedure: Add 10 mL hexane to column and place glass wool plug in bottom. Add 1 g silicic acid as hexane slurry, wash sides of column with hexane, and open stopcock to pack column. Top column with 1–2 cm Na_2SO_4. Drain most of hexane, and add 20 mL additional hexane to column. Add malt extract to column, using 5 mL hexane to wash concentrator tube and adding wash to column. Open stopcock and wash column with 50 mL DCM–pentane (4 + 10), elute NDMA with 50 mL DCM, and collect eluate in a Kuderna-Danish flask. Concentrate sample as in (a). (Recovery of NDMA was 80–89%.)

(c) One brand of bock beer was obtained from a retail source and the NDMA was isolated by a dry column procedure (17). The extract was passed through an acid-Celite column (18) followed by procedure (**b**). (Recovery of NDMA was 89%.)

(d) A 2 μL sample injected into the GC–MS instrument which was maintained in the direct inject mode 0.6 min, in the flush mode 0.6 min, and finally in the run mode. NPYR was determined on the UCON 5100 column, and NDMA was determined from malt and beer on the Carbowax 20M column. GC retention times were 22.8–23.0 min for NPYR on the UCON 5100 column, and 14.6 min for NDMA on the Carbowax 20M column.

Results and Discussion

Initially, the nitrosamine peak was collected as it eluted from the GC column and then analyzed by GC-MS (13), but this technique was too time consuming as a cleanup procedure and was abandoned for a column chromatographic method. Silica gel, as used in the FDA multidetection procedure for volatile nitrosamines (19), was also used here, but ethyl ether, one of the solvents specified to elute nitrosamines, could not be used here because solvent contaminants interfered with the GC-MS analysis. Silicic acid partially deactivated with 5% water was used for cleanup of all extracts containing NDMA because DCM eluted this nitrosamine, but not NPYR. Elution profiles of volatile nitrosamines on activity 1–3 acidic, basic, and neutral Woelm alumina have been investigated and summarized (20). Based on that work, neutral alumina of activity 3 was selected for our investigation. With this column, NDMA eluted with DCM–pentane (3 + 10) mixture, whereas NPYR eluted with a 6 + 10 mixture. Although NDMA and NPYR could be isolated separately with this column, no attempt was made to confirm NDMA in fried bacon because this nitrosamine is usually present in concentrations much lower than the 10 ppb violative level established by the regulatory agencies.

The criteria for this GC–MS confirmatory analysis for NDMA and NPYR are the simultaneous presence before, and absence after, UV photolysis of the principal characteristic ions monitored and the same GC retention time.

In low resolution mass spectra of NDMA and NPYR in which the ions from m/z 29 to 120 were scanned, ions with abundances greater than 10% of the base peak were m/z 30, 42, 43, and 74 (base peak) for NDMA, and m/z 30, 39, 41 (base peak), 42, 43, 68, and 100 for NPYR. Three characteristic and intense ions were selected for multiple ion analysis. These ions also gave the 3 most intense peaks in the ion chromatograms, namely, the parent ion, m/z 74 or 100, and m/z 30 (NO^+) and 42 ($C_2H_4N^+$). The latter 2 are common ion fragments of volatile nitrosamines (21). Although m/z 41 was more intense than m/z 42 for NPYR in the limited scan mass spectrum, this advantage was nullified by its greater prevalence from the fragmentation of some of the extract

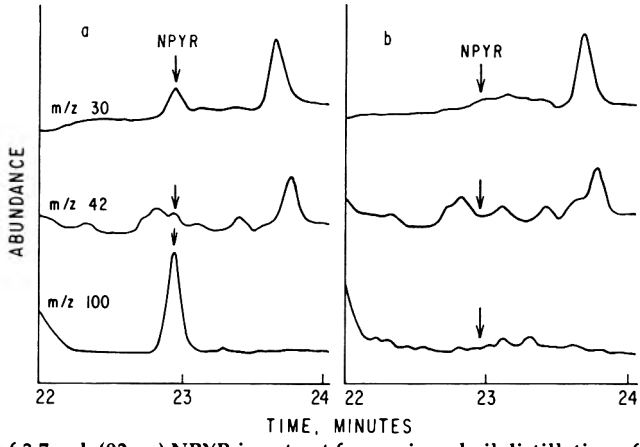


Figure 1. GC-MS of 3.7 ppb (92 ng) NPYR in extract from mineral oil distillation of fried bacon before (a) and after (b) UV photolysis.

contaminants when low levels of NPYR were analyzed.

The selected ion current profiles from the 3-ion monitoring of extracts from bacon and beer for NPYR or NDMA before (a) and after (b) photolysis under UV light are shown in Figures 1-3. The abundance scale for any given ion before and after photolysis differed by 20% or less except for m/z 100 in Figure 1, and m/z 74 for Figure 2, both of which differed by 70%, and for m/z 74 in Figure 3 which differed by 60%. The latter 3 are the result of the disappearance of the large nitrosamine peak after photolysis, with a corresponding decrease in the ion abundance scale. There were large variations in the ion abundance scale for different ions, therefore direct comparison of peak areas cannot be made.

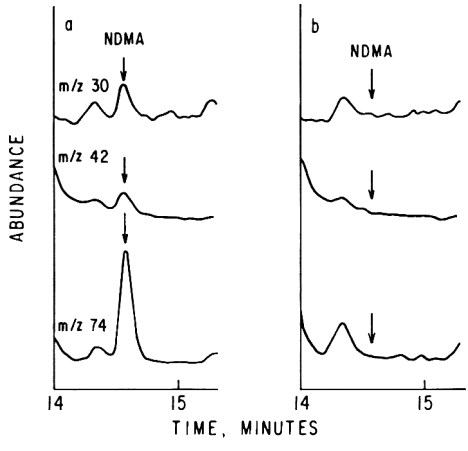


Figure 2. GC-MS of 1.5 ppb (73 ng) NDMA in extract from dry column treatment of beer before (a) and after (b) UV photolysis.

Bacon sample analyzed by the mineral oil distillation procedure and containing 3.7 ppb NPYR or a total of 92 ng NPYR is shown in Figure 1. The chromatogram represents an aliquot containing approximately 3 ng NPYR that was injected into the GC-MS system. The m/z 42 ion peak for NPYR is on the shoulder of a larger peak but it is evident that this as well as the other 2 peaks disappeared after photolysis. Two other peaks for m/z 42 at different retention times increased in size after photolysis, but did not interfere with the interpretation of the results. In fried bacon samples containing greater than 5 ppb NPYR, the intensity of all the ion peaks increased, particularly the m/z 42 peak. As the level of NPYR decreased to 5 ppb and below, the ions of m/z 42 and 30 for NPYR began to disappear into the background and became less discernible, while the m/z 100 ion peak remained strong because of the low background level for this ion. A more rigorous cleanup procedure than the one reported here should significantly decrease the background level, especially for m/z 42 and 30, thereby increasing the sensitivity of the procedure. When fried bacon was analyzed by the mineral oil distillation procedure, NPYR levels of 80-90 ng/25 g appeared to be the lower detection limit by this method. For fried bacon analyzed by the dry column method, extracts containing lower total NPYR than that from the mineral oil distillation procedure, 44 ng/10 g or 4.4 ppb, could be confirmed. The reasons were less ion fragments due to contaminants and therefore lower background levels for ions of

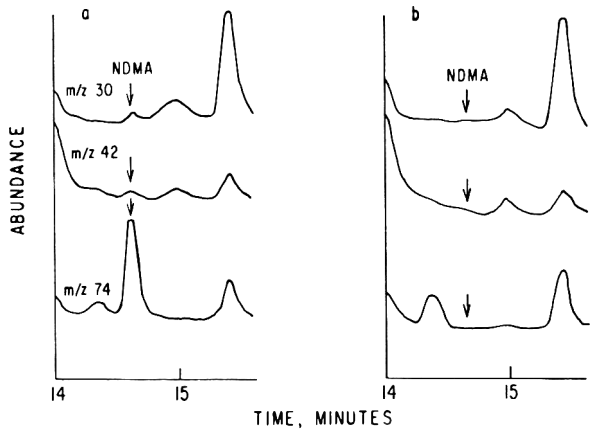


Figure 3. GC-MS of 2.8 ppb (71 ng) NDMA in extract from mineral oil distillation of malt before (a) and after (b) UV photolysis.

m/z 30 and 42, and probably less NPYR was lost during the concentration step. The tube containing the extract was cooled by the vaporization of the solvent during the concentration step and a thin film of solid, possibly mono- and diglycerides, which precipitated on the walls of the tube, melted at room temperature and was dissolved by washing the walls of the tube with the concentrate. This oily film probably decreased the loss of NPYR during the concentration step. On injection into the GC-MS system, these oils condensed on the walls of the injection port liner.

Figures 2 and 3 represent a bock beer extract containing 1.5 ppb or a total of 73 ng NDMA, and a malt sample analyzed by the mineral oil distillation procedure and containing 2.8 ppb NDMA or a total of 71 ng NDMA, respectively. In the chromatogram, which represents an injected sample containing approximately 2 ng NDMA, the presence before (a) and absence after (b) UV photolysis of NDMA is evident. The lower limit of detection for malt with the isolation procedure used was probably about 70 ng NDMA/25 g. For beer, the detection limit is not known but should be below 70 ng NDMA in the extract. The Food and Drug Administration recently established a NDMA violative level of 5 ppb for malt beverages (22) and >10 ppb for malt (23).

Extracts from malt contained a large peak that eluted just before NDMA on the UCON 5100 column, which interfered with the analysis. However, with the Carbowax 20M column, the elution time of this peak was about 1 min before the NDMA peak and could be eliminated from the ion chromatogram. The beer extract also contained interfering peaks that were present when the sample cleanup was performed by the acid-Celite or silicic acid columns, but the use of both columns eliminated this interference.

Ion peaks from non-nitroso and nonphotolyzable compounds were always present in the chromatograms. By using these peaks as internal reference standards, slight variations in the amount of the before and after photolysis samples injected into the GC-MS system, or abundance scale for a given ion chromatogram, did not interfere with the interpretation of the results.

This GC-MS procedure was applied successfully to the samples shown in Table 1. The greatest emphasis was placed on bacon because this was the foodstuff in which nitrosamines have been detected consistently. At present, fried bacon is routinely analyzed by the mineral oil distillation procedure for volatile nitrosamines; therefore, the greatest emphasis was placed on applying this GC-MS technique to extracts obtained by that isolation procedure. A few samples were analyzed by a recently developed dry column method (16) because this method appears to be more reliable and faster than the mineral oil distillation procedure and may find wider use for the routine analysis of NPYR in fried bacon.

The major advantage of this low resolution GC-MS confirmatory procedure is the low levels of nitrosamines required; the major disadvantage is that 2 analyses are required for the confirmation of one sample. This procedure will be suitable when the amount of sample is inadequate for a limited or full scan mass spectrum or a multiple ion analysis in which the ratios of the

Table 1. Samples used for GC-MS confirmatory method

Sample	Number	Wt of sample, g	Analytical method	Nitrosamine analyzed	Nitrosamine	
					Total, ng	ppb
Bacon	18	25	min. oil distn	NPYR	86–297	3.4–11.8
Bacon	3	10	dry column	NPYR	44–88	4.4–8.8
Malt	4	25	min. oil distn	NDMA	71–189	2.8–7.4
Beer	1	50	dry column	NDMA	73	1.5

ion peak areas are within a selected percent of the standard. The latter procedure requires a fairly clean sample; overlapping peaks such as is shown in Figure 1 (m/z 42) will interfere with integration of the peak area and thus the results. With the procedure reported in this paper, overlapping peaks will not be a problem and therefore the procedure can tolerate dirtier samples. One disadvantage of this procedure is that 2 very common ion fragments, m/z 30 and 42, are monitored. However, this disadvantage is overcome by the requirement of the simultaneous presence before and disappearance after UV photolysis, which is strong evidence that these ion fragments are actually from a nitrosamine and not another co-eluting compound.

The GC–MS confirmatory method for NDMA and NPYR should also be applicable to other volatile nitrosamines in other foods or nonfood substrates. This technique can also be applied to obtain a full-scan mass spectrum when sufficient concentrations of nitrosamines are available in the presence of co-eluting non-nitrosamines by subtracting the full-scan mass spectrum obtained after photolysis from the one before photolysis.

Acknowledgment

We thank T. Phillippo of the Food Safety and Inspection Service, U.S. Department of Agriculture, for fried bacon samples; D. C. Havery of the Food and Drug Administration, Health and Human Services, for malt samples; and the National Cancer Institute for the loan of a thermal energy analyzer under contract No. N01-CP-55715.

References

(1) Magee, P. M., & Barnes, J. M. (1967) *Adv. Cancer Res.* **10,** 163–246
(2) Gough, T. A. (1978) *Analyst* **103,** 785–806
(3) Gough, T. A. (1978) in *Environmental Carcinogens Selected Methods of Analysis. Volume 1—Analysis of Volatile Nitrosamines in Food*, H. Egan (Ed.), Scientific Publication No. 18, International Agency for Research on Cancer, Lyon, France, pp. 29–34
(4) Sphon, J. A. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1247–1252
(5) Fazio, T., Damico, J. N., Howard, J. W., White, R. H., & Watts, J. O. (1971) *J. Agric. Food Chem.* **19,** 250–253
(6) Sen, N. P., Schwinghammer, L. A., Donaldson, B. A., & Miles, W. F. (1972) *J. Agric. Food Chem.* **20,** 1280–1281
(7) Havery, D. C., Hotchkiss, J. H., & Fazio, T. (1980) *J. Food Sci.* **46,** 501–505
(8) Fazio, T., Havery, D. C., & Howard, J. W. (1981) in *N-Nitroso Compounds: Analysis, Formation and Occurrence*, E. A. Walker, L. Griciute, M. Castegnaro, & S. M. Börzsöngi (Eds), Scientific Publication No. 31, International Agency for Research on Cancer, Lyon, France, pp. 419–433
(9) Hotchkiss, J. H., Libbey, L. M., & Scanlan, R. A. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 74–79
(10) Sander, J. (1967) *Hoppe-Seylers Z. Physiol. Chem.* **348,** 852–854
(11) Fan, T. Y., & Tannenbaum, S. R. (1971) *J. Agric. Food Chem.* **19,** 1267–1269
(12) Doerr, R. C., & Fiddler, W. (1977) *J. Chromatogr.* **140,** 284–287
(13) Kimoto, W. I., Dooley, C. J., Carré, J., & Fiddler, W. (1981) *Water Res.* **15,** 1099–1106
(14) Pensabene, J. W., Fiddler, W., Miller, A. J., & Phillips, J. G. (1980) *J. Agric. Food Chem.* **28,** 966–970
(15) Fine, D. H., Rufeh, F., Lieb, D., & Rounbehler, D. P. (1975) *Anal. Chem.* **47,** 1188–1191
(16) Pensabene, J. W., Miller, A. J., Greenfield, E. L., & Fiddler, W. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 151–156
(17) Hotchkiss, J. H., Havery, D. C., & Fazio, T. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 929–932
(18) Howard, J. W., Fazio, T., & Watts, J. O. (1970) *J. Assoc. Off. Anal. Chem.* **53,** 269–274
(19) Fazio, T., Howard, J. W., & White, R. (1972) in *N-Nitroso Compounds Analysis and Formation*, P. Bogovski, R. Preussman, & E. A. Walker (Eds), Scientific Publication No. 3, International Agency for Research on Cancer, Lyon, France, pp. 16–24
(20) Telling, G. M. (1972) *J. Chromatogr.* **73,** 79–87
(21) Lijinsky, W., Christie, W. H., & Rainey, W. T. (1973) in *Mass Spectra of N-Nitroso Compounds*, Oak Ridge National Laboratory Report ORNL-TM-4359, pp. 1–106
(22) *Fed. Regist.* (1980) **45,** 39341
(23) *Fed. Regist.* (1981) **46,** 39218–39219

ANTIBIOTICS

Cylinder Plate Assay for Determining Bacitracin in Premix Feeds and Finished Feeds: Collaborative Study

JOHN B. GALLAGHER and LINDA L. KNOTTS
International Minerals & Chemical Corp., PO Box 207, Terre Haute, IN 47808

Collaborators: D. Brennecke; H. H. Bryant; G. G. Carter; C. Harpster; M. L. Hasselberger; K. D. Jones; L. L. Knotts; J. Martin; J. I. Olsen; H. S. Ragheb; N. Stouffer; C. Winely

A cylinder plate assay procedure was studied by 10 laboratories. For premix feeds, 3 samples of bacitracin methylene disalicylic acid and 3 samples of bacitracin zinc premixes covering the range of 10 to 50 g/lb were used. The repeatability standard deviation was 2.11, and the reproducibility standard deviation was 2.13. The average recovery of bacitracin was 101.5%. The method has been adopted official first action. For finished feeds, 6 samples of bacitracin methylene disalicylic acid and 6 samples of bacitracin zinc covering the range of 10 to 800 g/ton were used in the study. The procedure included a sample cleanup step using disposable reverse phase columns. This step appears to be the cause of the poor results reported by most collaborators. Continued study is needed to develop an acceptable method for finished feeds.

Bacitracin is a polypeptide antibiotic produced by *Bacillus lichenformis* and consists of 3 active components. Bacitracin added to animal feeds for growth promotion, feed efficiency, and disease control is stabilized by reaction with zinc or methylene disalicylate (MD). It can be determined in premix feeds in the concentration range 10–50 g/lb and in finished feeds in the range 10–1000 g/ton by a high pressure liquid chromatographic procedure (1) or by biological methods (2–4). The biological assay procedures developed for this study were collaboratively tested because most state laboratories already have the necessary equipment and qualified personnel necessary to perform microbiological assays.

The AOAC official first action method for finished feeds uses an acidified pyridine extraction procedure followed by determination using the *Micrococcus flavus* cylinder plate assay. Ragheb proposed and studied a methanol extraction procedure with determination by using the cylinder plate assay technique.

Work in the Associate Referee's laboratory supports Ragheb's work which showed a positive bias by the pyridine procedure. Also, in the development of the procedure for collaborative study, it was noted that in many instances without sample cleanup, both positive and negative bias existed with the methanol procedure.

Collaborative Study

The 3 statistical unit blocks used for the premix study consisted of commercial premix level feeds with nominal potencies of 10, 25, and 50 g/lb. One member of each unit block consisted of a zinc and the other a methylene disalicylate bacitracin premix.

Bacitracin feed premixes are biomass products that do not have a known concentration of bacitracin. To evaluate method recovery, the 25 g/lb unit block was prepared in the Associate Referee's laboratory as a 1 + 1 dilution of the respective 50 g/lb premixes. A V-notch blender was used to ensure a homogeneous blend.

The 6 statistical unit blocks used for the finished feed study were prepared at nominal concentrations of 10, 15, 100, 150, 500, and 800 g/ton from both poultry and swine unmedicated premixes. One member of each unit block consisted of a zinc and the other a methylene disalicylate bacitracin. Five-pound batches of each member of each unit block were prepared by accurately weighing the poultry or swine premixes and then adding the appropriate amount of zinc or methylene disalicylate bacitracin premix. Each batch was blended 20 min in a V-notch blender and then ground to a consistent particle size.

Materials for each unit block were riffled and packaged in moisture-proof containers. Six containers of each member of each unit block

This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee G and adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) 65, 389.

Received August 21, 1981. Accepted March 29, 1982.

0004-5756/82/6505-1168-10$01.00

were analyzed to confirm the homogeneity of the packaged samples. Each package was labeled with the nominal potency and a unique set of random numbers for each collaborator.

The 11 collaborators were sent 2 samples of each member of each unit block and were asked to perform a single analysis on each of the 12 premix samples and 24 finished feed samples. Collaborators were supplied with 1 g zinc bacitracin standard, a slant of *M. flavus* ATCC 10240 culture, a pneumatic compeller, a copy of the method, photos showing use of the pneumatic compeller and preparation of the cleanup column, glass filter pads used in the preparation of cleanup columns, and report forms.

Collaborators were also asked to prepare 2 additional dilutions of the standard solution and to plate these as samples to evaluate the standard curve error.

Pretest of the Method.—In advance of the collaborative study, the method was subjected to critical review and testing by H. S. Ragheb of Purdue University. Problems encountered were reviewed and corrected. In addition, the following parameters were tested in the Associate Referee's laboratory: extraction time, effect of organic extraction solvent on *M. flavus*, evaporation pH, analytical error expected with various plate sensitivities, number of plates that should be used in each determination, cleanup column configuration, washing solvents for the cleanup columns, and elution volume for bacitracin from cleanup columns.

A minimum of 5 min is required to obtain complete extraction. The plate sensitivity was adjusted to eliminate interference from organic solvents. Interaction of the error associated with the selected plate sensitivity and the number of plates required to provide a consistent error led to the conclusion that 4 plates were required for each determination.

The pH adjustment prior to evaporation was found to be a critical factor in the finished feed analysis. Methanol was selected as the elution solvent and the 3.5 mL elution volume was selected to provide a safety factor of 1 mL for bacitracin elution.

Bacitracin in Premix Feeds

Cylinder Plate Assay

Official First Action

(Applicable to premixes contg ≥10 g bacitracin/lb)

42.C06 *Principle*

Bacitracin is extd from feeds into acidified org. solv. system. Ext is centrfd, and supernate is dild in phosphate buffer and analyzed by cylinder plate assay with *M. flavus* as detection organism.

42.C07 *Reagents and Apparatus*

(a) *Microorganism.*—*Micrococcus flavus* ATCC 10240. Maintain culture as indicated in **42.199(a)**.

(b) *Extracting solv.*—Mix, by vol., 27% CH_3CN, 27% MeOH, 3% pH 6.0 phosphate buffer, (**42.197**)(**f**), 41% H_2O, and 2% H_3PO_4 (85%); add 0.5 g EDTA/L. (Extg solv. is satd with EDTA.).

(c) *Phosphate buffer.*—5%, pH 6.5. See **42.197(d)**.

(d) *Diluting solvent.*—Methanol–5% pH 6.5 phosphate buffer (12 + 88).

(e) *Dilute HCl.*—Carefully add 89 mL HCl to H_2O and dil. to 1 L (1N). Further dil. soln 1:100 (0.01N).

(f) *Cylinders.*—See **42.198(a)**.

(g) *Cylinder dispenser.*—Optional: see **42.198(c)**.

42.C08 *Standard Solutions*

See **42.202(a)** and (**b**). Also prep. 0.30 and 0.16 unit/mL solns to be plated as samples to monitor assay.

42.C09 *Preparation of Plates*

Use one layer (ca 15 mL) of agar antibiotic medium 1, **42.196(a)**. Det. by trial plates optimum concn (usually 0.02–0.05%) of *M. flavus* ATCC 10240 to be added to agar to obtain zones of inhibition 15–17 mm for 0.2 unit bacitracin/mL. Pour 4 plates for each point on std curve (i.e., 16 plates) and 4 plates for each sample soln. Std curve will be plated twice (i.e., 32 plates) as will check samples 0.30 and 0.16 unit/mL. Therefore, total of 48 plates will be needed for 2 curves and check samples, plus 4 addnl plates for each sample.

Let agar harden on level surface. Transfer to refrigerator and cool ≥1 h before dosing. Use plates same day prepd.

42.C10 *Extraction*

Accurately weigh amt feed contg ca 4600 units of bacitracin into 300 mL erlenmeyer flask, or equiv.

Add 100 mL extg solv. with 100 mL vol. pipet and ext feeds ≥5 min by shaking flask or mixing on mag. stirrer.

Transfer supernate to plastic centrif. tubes and centrif. 10 min at 2000 rpm. Filter supernate thru glass wool into graduate. Use vol. glassware and dilg solv. to prep. final diln 0.2 ± 0.05 unit/mL.

42.C11 ***Plating***

Use 16 seeded plates for first curve. Use 0.20 unit/mL as plate ref. On each plate, fill 3 alternate stainless steel cylinders with plate ref. and the 3 remaining cylinders with 1 std. Be sure all cylinders are filled with const vol. (i.e., 0.25 mL). Preset Eppendorf pipet is best for this purpose. Use 4 plates for each sample, including 0.3 and 0.16 unit/mL check samples.

Use 16 seeded plates for second curve, to be plated after all samples are plated. Use 8 plates for second plating of 0.3 and 0.16 unit/mL check samples.

Incubate dosed plates 16–18 h at 37 ± 2°. Read zones of inhibition to nearest mm, using Fisher-Lily zone reader.

42.C12 ***Determination***

Det. corrected av. zone diams for std (Z′) and sample (Z) solns according to **42.200.** Det. response line as least squares linear regression of following equation:

$$Z' = m\log P' + b$$

where P' = potency in unit/mL of std soln associated with Z'; m,b = are least squares fitted slope and intercept parameters. Calc. potency of sample by following equation:

$$\text{g bacitracin/lb} = [\text{antilog}(Z - b/m) \times D \times 0.0108]/\text{sample wt}$$

where D = total sample diln; 0.0108 = 453.6 (g/lb)/42 000 (units/g bacitracin).

Bacitracin in Finished Feeds

Principle

Feeds are washed with petroleum ether to remove fat before extraction into acidified organic solvent system. Extract is then centrifuged and filtered through glass wool, pH is adjusted, and organic solvent is removed. Residue is diluted with buffer, centrifuged, and applied to cleanup column. Eluate is analyzed by cylinder plate assay using *M. flavus* as detection organism.

Reagents and Apparatus

See **42.C07,** plus the following:

(a) *Rinsing solvent.*—Mix, by volume, 44% water, 44% pH 6.5 phosphate buffer (5%), and 12% methanol.

(b) *Pneumatic compeller.*—These devices were supplied by the Associate Referee (Figure 1).

Standard Solution

See **42.C08.**

Preparation of Plates

See **42.C09.**

Cleanup Column

Prepare cleanup columns from two 3 mL plastic syringes. Remove plunger tips and save. Cut Luer-lok tip from syringe and discard remainder. Place Type AP (No. AP25 01000) Millipore filter in bottom of second syringe. Slurry LiChroprop RP-8 packing (EM reagent No. 9324-4R) in methanol; then draw into syringe until packing reaches 20 mm mark. Place another Type AP Millipore filter on top of packing. Insert 1½ in. 18G needle through Luer-lok tip cut from first syringe and push 2 plunger tips onto needle. Insert this assembly into top of second syringe. Figure 2 is a sketch of finished mini column. Prepare column for use by pumping 5 mL methanol followed by 5 mL rinsing solvent through column.

Extraction

One day before assay, extract feeds in Goldfisch apparatus to remove fat: Accurately weigh amount of feed containing 20 units of bacitracin into folded Whatman filter paper. Place in stainless steel thimble. Reflux 1 h on high temperature setting. Use 40 mL petroleum ether as solvent. Remove feed and let air-dry overnight.

On day of assay, carefully transfer treated feed to 300 mL Erlenmeyer flask or equivalent. Add 100 mL extracting solvent, using 100 mL volumetric pipet. Extract feed 5 min by either shaking flask or using magnetic stirrer. Transfer supernate to centrifuge tubes and centrifuge 10 min at 2000 rpm. Filter supernate through glass wool into graduated cylinder.

Using volumetric pipet, remove appropriate volume (containing 5 units of bacitracin) and place in side-arm filtering flask. Adjust pH to 6.5 with 5N NaOH. Sparge off organic material by using stream of air into side-arm flask. Organic solvent is gone when no methanol or acetonitrile odor remains. The usual time for this process is 20 min.

Transfer liquid from side-arm flask to centrifuge tube. Quantitatively wash side-arm flask

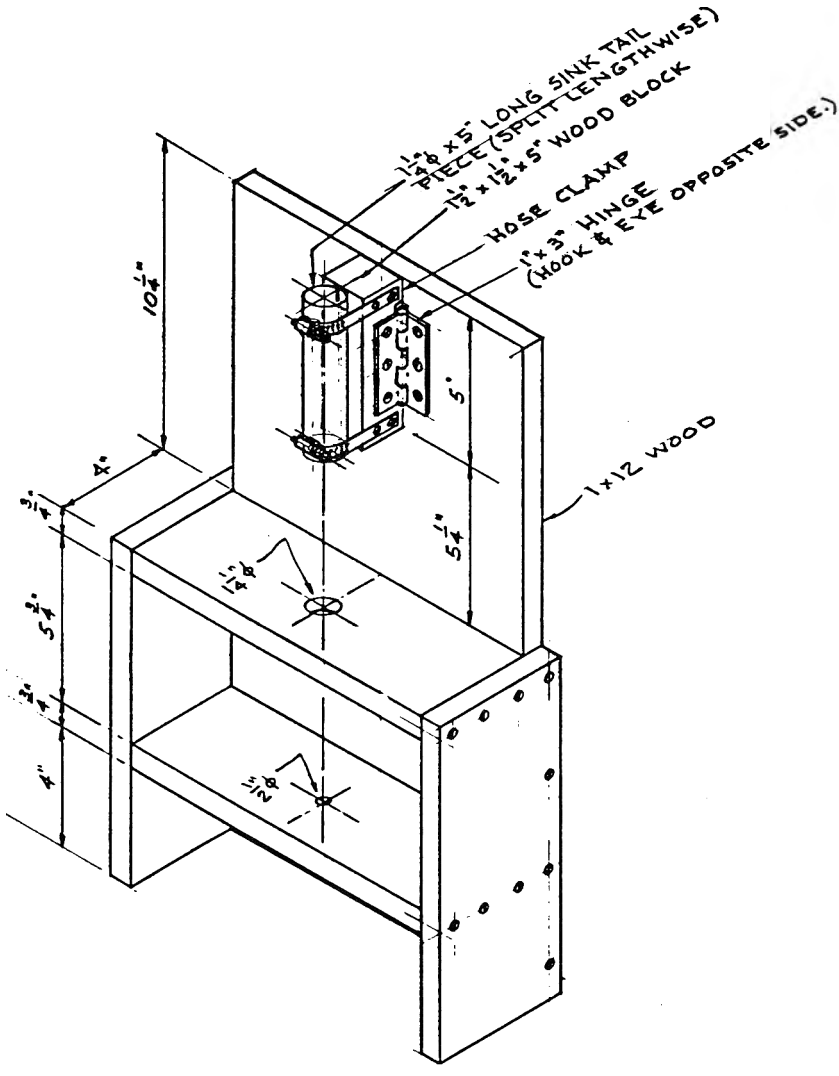


Figure 1. Pneumatic compeller.

with 10 mL rinsing solvent. All solids must be resuspended in solvent; ultrasonic bath is useful for this purpose. Transfer first wash to centrifuge tube containing supernate from sparging process. Again wash side-arm flask with 10 mL rinsing solvent. Transfer second wash to centrifuge tube. Add final 10 mL rinsing solvent to side-arm flask and set aside. The third wash will be used to rinse column. Centrifuge sparged material and the 20 mL rinsing solvent 5 min at 1200 rpm.

After centrifugation, carefully transfer supernate to 50 mL disposable syringe. Attach to prepared cleanup column. Apply air pressure by using pneumatic compeller (Figure 1). Air pressure must be ca 40 psi but should not exceed 60 psi. Let liquid be forced through column to 1/4 in. above glass fiber filter on top of packing. Do not let column dry during this process.

Remove 50 mL syringe from pneumatic compeller. Carefully transfer third and final wash from side-arm flask to syringe. Attach to compeller and force liquid through column to 1/4 in. above glass fiber filter. Do not let column dry. Remove column from compeller. Fill 3 mL disposable syringe with 3.5 mL (2 mL plus 1.5 mL) of 100% methanol. Attach to top of column and collect effluent in 25 mL volumetric flask. Fill

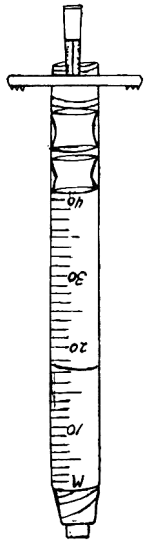


Figure 2. Sketch of completed mini column.

flask to volume with pH 6.5 phosphate buffer (5%) and mix well. Let foam subside, pour test solutions into glass tubes, and submit for plating. Use only glass tubes because plastic tubes such as polystyrene can interfere with assay.

Plating

See 42.C11.

Determination

See 42.C12.

Results and Discussion – Premix Feeds

Collaborative results were received from 10 of the 11 laboratories that were sent samples for analysis. Results are presented in Table 1. Samples 1, 3, and 5 are zinc bacitracin premixes; Samples 2, 4, and 6 are MD premixes. The design of the study allows the application of both the analysis of variance procedure of Steiner and the method of Youden for statistical analysis of collaborative studies (5).

The 2-sample charts for the 3 unit blocks are presented in Figure 3. As usual, the points are found predominantly in quadrants I and III, representing +,+ and −,− results: 67% of the results lie in quadrants I and III, 33% appear in quadrants II and IV. The points are generally arranged in the usual elliptical pattern around a major axis at 45°. Laboratory 3 which obtained the lowest laboratory rank was located consistently in quadrant III and was outside the 95% confidence circle, indicating a large systematic error. However, in 2 of the unit blocks reported by Laboratory 3, the results lie near the 45° line, indicating good precision for the majority of data received from this laboratory. Laboratory 10 which had the highest laboratory score was located consistently in quadrant I and was well outside the 95% confidence circle, indicating a very large systematic error. However, each unit block reported by Laboratory 10 was near the 45° line, indicating good precision. In general, these plots show that the method does not have an extremely high precision. However, such observations are typical for microbiological assays.

Table 2 presents the results of the extra standard dilutions that were plated as samples. These results again indicate that Laboratory 10 had a systematic problem with the procedure. Laboratory 3 showed a low recovery of the standard solution, which is consistent with the conclusion drawn from 2-sample charts. Laboratory 2 also showed a low recovery on the standard and had a low laboratory rank.

Rejection of Outliers.—The results of ranking the collaborative analysis are presented in Table 3. Laboratory 10 ranked highest on all 6 samples, and Laboratory 3 ranked lowest on 5 of 6 samples. Total ranks for Laboratories 10 and 3 are well outside the approximate 5% 2-tail limits

Table 1. Replicate results from collaborative tests reported by 10 laboratories on 6 premix samples (g/lb)

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
Lab.	Replicates	Sum	Replicates	Sum	Replicates	Sum	Replicates	Sum	Replicates	Sum	Replicates	Sum
1	10.8, 12.1	22.9	10.0, 9.3	19.3	28.1, 26.1	54.2	23.2, 21.7	44.9	57.4, 51.7	109.1	46.2, 48.3	94.5
2	9.7, 11.3	21.0	9.7, 9.0	18.7	30.9, 21.9	52.8	23.9, 23.9	47.8	56.0, 52.3	108.3	44.5, 46.3	90.8
3	9.2, 10.6	19.8	7.5, 8.1	15.6	23.8, 25.6	49.4	20.9, 19.3	40.2	50.3, 50.0	100.3	43.5, 43.4	86.9
4	10.5, 12.8	23.3	9.9, 9.4	19.3	25.2, 31.3	56.5	22.4, 24.8	47.2	56.5, 60.4	116.9	48.4, 43.7	92.1
5	11.7, 10.7	22.4	10.3, 9.4	19.7	29.6, 31.5	61.1	22.0, 21.1	43.1	53.0, 55.1	108.1	44.5, 46.5	91.0
6	11.1, 12.0	23.1	8.7, 10.5	19.2	29.3, 28.9	58.2	24.3, 25.2	49.5	54.3, 54.3	108.6	44.6, 48.7	93.3
7	10.7, 9.9	20.6	8.5, 9.2	17.7	28.9, 28.8	57.7	21.2, 22.7	43.9	53.6, 57.7	111.3	44.4, 41.2	85.6
8	11.4, 11.5	22.9	9.5, 10.6	20.1	29.2, 28.3	57.5	22.6, 21.9	44.5	57.4, 55.2	112.6	36.4, 47.7	84.1
9	10.6, 11.2	21.8	8.3, 8.3	16.6	26.6, 26.8	53.4	22.0, 22.2	44.2	55.0, 55.2	110.2	45.1, 46.6	91.7
10	16.8, 13.9	30.7	11.4, 12.2	23.6	30.8, 32.4	63.2	37.0, 30.8	67.8	64.5, 84.0	148.5	58.1, 55.4	113.5

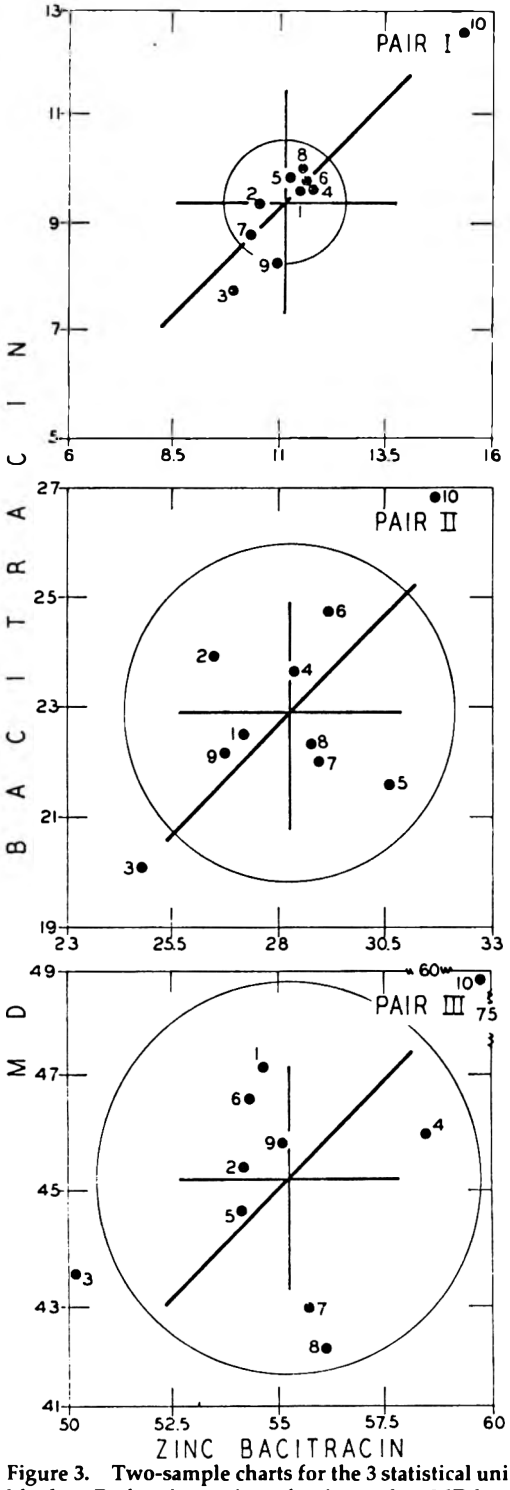


Figure 3. Two-sample charts for the 3 statistical unit blocks. Each pair consists of a zinc and an MD bacitracin premix. Numbers beside points identify collaborators. Average values are indicated by cross-hatch. Circle represents 95% confidence interval around average.

for 10 laboratories with 6 samples of 14 and 52. As a result of the ranking of these laboratories and their consistent presence in quadrants I and III of the 2-sample charts, data from these laboratories were excluded from statistical analysis.

Table 4 presents the results of the Dixon test conducted on the sum of duplicates to determine individual outlying results. None of the calculated values exceeded the 1 in 20 probability of 0.55 and therefore were not rejected from analysis.

Homogeneity of Variation.—The homogeneity of experimental variation between laboratories was evaluated based on the range of duplicate results on each sample. The ranges, W_s, for S = Samples 1-6 are 2.7, 3.5, 8.3, 6.4, 8.8, and 10.4 g/lb, respectively. This gives an experimental ratio W_{max}/W_{total} = 10.4/40.1 = 0.26, which is below the 1 in 20 significance level of 0.28. Therefore, there is no statistical evidence that the samples need to be analyzed separately.

The homogeneity of experimental variations between replicates was evaluated using the maximum and sum of the ranges ratio test. The result was significant at the 5% significance level. However, the data were not rejected because of the abnormal low sum obtained on Samples 1 and 2 which adversely weighted the evaluation, and because the average results on the data were near results reported by other collaborators.

Statistical Results.—Table 5 summarizes the results of the statistical analysis conducted by the Youden method of analysis of variance. The precision standard deviation, S_r, was computed from the difference of the average result of paired samples, correcting S_r by $\sqrt{2}$; the distribution standard deviation, S_d, from the total of the pairs; and the standard deviation for the distribution of systematic error, S_b, from $S_d^2 = 2 S_b^2 + S_r^2$. The calculated F-values in Table 4 are less than the critical F-value at the 10% level of significance for 8 degrees of freedom. Hence, the systematic error, S_b, does not make a statistically significant contribution ($P > 0.1$) to the standard deviation of the data S_d.

Table 6 summarizes the results of the statistical analysis conducted using the nested analysis of variance technique of Steiner. The between-laboratory variance ratio is 1.22 and is not significant at the 10% level of significance. Nor is the laboratory-sample error significant at the 10% level. The repeatability standard deviation (within-laboratory) estimate is 2.13 g bacitracin/lb. The reproducibility standard deviation (between-laboratories) estimate is 2.15 g bacitracin/lb. These values correspond to the

Table 2. Percent recovery on 0.16 and 0.30 unit/mL bacitracin standard

	Laboratory										
Std	1	2	3	4	5	6	7	8	9	10	Av.
0.16	95.4	89.7	92.0	95.3	97.8	94.4	85.6	97.1	97.8	113.8	95.89
0.30	104.4	93.1	91.8	96.7	95.8	102.0	119.0	98.0	101.2	114.0	101.60
Av.	99.9	91.4	91.9	96.0	96.8	98.2	102.3	97.6	99.5	113.9	98.75

Table 3. Ranking of premix data from Table 1

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6		
Lab.	Sum	Rank	Sum	Rank	Sum	Rank	Sum	Rank	Sum	Rank	Sum	Rank	Total Rank
1	22.9	6.5	19.3	6.5	54.2	4.0	44.9	6.0	109.1	5.0	94.5	9.0	37.0
2	21.0	3.0	18.7	4.0	52.8	2.0	47.8	8.0	108.3	3.0	90 8	4.0	24.0
3	19.8	1.0	15.6	1.0	49.4	1.0	40.2	1.0	100.3	1.0	86.9	3.0	8.0
4	23.3	9.0	19.3	6.5	56.5	5.0	47.2	7.0	116.9	9.0	92.1	7.0	43.5
5	22.4	5.0	19.7	8.0	61.1	9.0	43.1	2.0	108.1	2.0	91.0	5.0	31.0
6	23.1	8.0	19.2	5.0	58.2	8.0	49.5	9.0	108.6	4.0	93.3	8.0	42.0
7	20.6	2.0	17.7	3.0	57.7	7.0	43.9	3.0	111.3	7.0	85.6	2.0	24.0
8	22.9	6.5	20.1	9.0	57.5	6.0	44.5	5.0	112.6	8.0	84.1	1.0	35.5
9	21.8	4.0	16.6	2.0	53.4	3.0	44.2	4.0	110.2	6.0	91.7	6.0	25.0
10	30.7	10.0	23.6	10.0	63.2	10.0	67.8	10.0	148.5	10.0	113.5	10.0	60.0

equivalent values S_b and S_d obtained by the Youden procedure.

Recovery.—Because of the biomass nature of bacitracin premix products, it is possible to obtain only a method recovery. Sample pair II was specially prepared by the Associate Referee from sample pair III to provide a method of estimation of the method accuracy. Table 7 presents the results of the calculated recovery obtained by each collaborator on both methylene disalicylate and zinc bacitracin feeds. These values were obtained by the following formulas:

$$\% \text{ Recovery } MD_i = (\text{av. value Sample 4})_i \times 2/(\text{av. value Sample 6})_i$$

$$\% \text{ Recovery zinc}_i = (\text{av. value Sample 3})_i \times 2/(\text{av. value Sample 5})_i$$

where i represents each collaborator.

The recovery values for MD bacitracin range from 94.7 to 106.1% with an average value of 101.2 ± 4.2%. Recovery values for zinc bacitracin range from 96.7 to 113.0% with an average value of 103.1 ± 4.8%. By Student's test ($t_{exp} = 0.29$), the 2 materials have equivalent recoveries ($P > 0.1$). Overall average recovery for the method is 101.6 ± 2.8%.

Comments of Collaborators.—One collaborator was concerned with the exposure of laboratory personnel to acetonitrile. The preparation of the extraction solvent should be carried out in a hood as dictated by safe laboratory practices. One collaborator had an abnormal loss in moisture during drying of the standard. This collaborator was sent fresh samples and redid the assays. One collaborator added neomycin to the agar to enhance zone size. No major problems with the technique were noted by any collaborators.

Table 4. Outlying individual results in premix study

	Sample					
Value[a]	1	2	3	4	5	6
Highest	0.09	0.17	0.38	0.30	0.50	0.13
Lowest	0.16	0.35	0.11	0.17	0.04	0.16

[a] Critical value with 1 in 20 probability is 0.55; critical value with 1 in 100 probability is 0.68.

Results and Discussion – Finished Feeds

Collaborative results were received from 10 of the 11 laboratories sent samples for analysis. Results of the collaborative study are presented in Table 8. Samples 4, 5, and 6 were prepared from zinc bacitracin and an unmedicated poultry premix; Samples 10, 11, and 12 were prepared from zinc bacitracin and an unmedicated swine premix. Samples 1, 2, and 3 were prepared from MD bacitracin and an unmedicated poultry premix; Samples 7, 8, and 9 were prepared from MD bacitracin and an unmedicated swine premix.

Table 9 shows the results of ranking the laboratories by random selection of one value for each laboratory on each sample. Results are

Table 5. Results of statistical analysis by Youden method—premix study

Pair	Av. bacitracin, g/lb		Precision	Systematic	Distribution		
	Zinc	MD	S_r	S_b	S_d	S_d^2/S_r^2	DF
I	11.12	9.41	0.470	0.356	0.689	2.14	7
II	28.28	22.82	1.989	—	1.118	0.32	7
III	55.32	45.19	2.642	—	1.432	0.29	7

Table 6. Analysis of variance—premix study

Source of variance	Sum of sq.	DF	Mean sq.	Variance ratio
Between laboratories	0.25672E + 02	7	3.667	1.219
Between samples	0.27141E + 05	5		
Lab-sample interaction	0.10532E + 03	35	3.009	0.661
Between replicates	0.21839E + 03	48	4.550	
Total	0.27490E + 05	95		

within the 2-tail 95% confidence limits for 9 laboratories with 12 samples.

Table 10 shows the theoretical values for each feed and the average recoveries. It is obvious from these data that the method does not recover all bacitracin added to the samples.

Table 11 presents an analysis of variance on the data in Table 9. The calculated *F*-ratio is significant at 1 in 20 significance level (critical value 2.9). In addition, data in Table 8 show a high precision error, although it was not possible to statistically estimate its value. Between-laboratory error was estimated from Table 11 to be 53 g bacitracin/ton.

Collaborators' Comments.—Collaborators had difficulty understanding the column preparation and use of the pneumatic compeller, even though illustrations were sent to each collaborator. Many collaborators complained about the time required for sample preparation. However, it has been determined by the Associate Referee that sample cleanup is necessary to obtain accurate results.

Conclusions and Recommendations

The poor recovery and precision obtained in the finished feeds study resulted from the lack of experience of many of the collaborators with the cleanup techniques used for trace analysis. The Associate Referee should have provided practice samples to allow technique development. It is recommended that study of the microbiological assay technique for the determination of bacitracin in finished feeds be continued.

In a separate paper, a collaborative study using the same unit blocks was conducted of a liquid chromatographic assay for premixes (6). The results of the 2 collaborative studies compare statistically and provide support that both methods provide accurate and precise results. Analysis of the data by the 2 statistical procedures recommended by AOAC provides added evidence of the validity of the procedure for premixes. The Associate Referee recommends adoption of this microbiological assay as official first action.

Acknowledgments

The Associate Referee acknowledges the special efforts of L. L. Spittler for her assistance in the preparation of this manuscript; H. S. Ragheb for the preliminary study of the method and helpful suggestions; C. Harpster for her assis-

Table 7. Calculated percent recovery obtained by collaborators—premix study

Bacitracin	Laboratory								Av.
	1	2	3	4	5	6	7	8	
MD	95.0	105.3	103.7	94.7	106.1	102.6	105.8	96.4	101.2 ± 4.2
Zinc	99.3	97.5	96.7	113.0	107.2	103.7	102.1	96.9	102.0 ± 4.8
Overall recovery									101.6 ± 2.8

Table 8. Results from collaborative study of cylinder plate assay for determining bacitracin in finished feeds (g/ton)

	Sample											
Lab.	1	2	3	4	5	6	7	8	9	10	11	12
1	9.0[a]	86.7[a]	351.1[a]	16.0[a]	144.2[a]	710.2	7.4[a]	70.4	399.6[a]	13.1	124.9[a]	636.7[a]
	—[b]	72.3	384.1	12.3	135.1	760.1[a]	7.9	92.7[a]	374.5	16.3[a]	146.9	714.7
2	7.5[a]	75.9[a]	357.3	14.2	143.2	739.1[a]	4.6	75.0[a]	359	9.6[a]	37.8	535.8[a]
	5.0	82.0	408.9[a]	13.0[a]	159.9[a]	740.9	4.9[a]	69.4	334.4[a]	10.7	126.6[a]	661.0
3	1.9[a]	53.2	379.9[a]	12.7[a]	124.5[a]	654.5	4.0[a]	69.9	354.8[a]	13.7[a]	86.6	788[a]
	—	63.5[a]	163.8	14.5	170.1	823.5[a]	—	92.4[a]	371.8	—	150.7[a]	812
4	5.2	66.3[a]	290.0	10.2	130.3	606.4[a]	6.3	64.5[a]	385.3	13.2[a]	131.4[a]	575.7[a]
	5.7[a]	60.2	326.2[a]	12.0[a]	108.2[a]	543.6	6.7[a]	—	352.1[a]	16.7	135.3	566.3
5	1.2	43.2	262.1[a]	7.3[a]	79.2[a]	510.2	—	46.1	258.7[a]	10.7[a]	89.0[a]	478.2
	3.6[a]	54.5[a]	—	4.9	110.2	468.4[a]	3.9[a]	44.4[a]	—	7.5	79.6	506.6[a]
6	3.5[a]	66.7[a]	—	—	95.3	500.2[a]	5.1[a]	71.4[a]	457.2[a]	10.0	75.0	657.6[a]
	4.2	45.3	276.4[a]	4.4[a]	95.2[a]	542.4	5.3	66.2	330.7	11.5[a]	116.8[a]	148.7
7	3.4	49.4	358.3[a]	7.9[a]	137.0[a]	695.3	4.7	60.6	236.5[a]	11.3	125.0[a]	704.6[a]
	4.1[a]	72.1[a]	457.4	9.0	132.9	695.4[a]	5.3[a]	70.1[a]	351.9	11.4[a]	104.7	601.5
8	2.4	—	—	—	35.2	302	7.8	70.4	110.2	15.2	—	292.7
	2.6	130.7	—	—	—	—	—	—	—	—	—	587.9
9	4.0[a]	74.9[a]	162.6	5.3[a]	86.6[a]	554.7[a]	1.5	77.7[a]	238.9[a]	9.6[a]	122.2[a]	608.5
	—	—	224.2[a]	—	—	411.8	1.3[a]	—	129.0	—	—	505[a]
10	1.7[a]	45.8	347.1[a]	6.2	147.3[a]	649.2	3.7[a]	69.5	265.7	6.9	129.3	563.6[a]
	—	68.6[a]	220.8	8.1[a]	137.4	567.0[a]	4.0	69.0[a]	346.8[a]	10.8[a]	145.4[a]	499.5

[a] Used in statistical analysis.
[b] No result reported.

Table 9. Ranking of results from Table 8

	Sample												
Lab.	1	2	3	4	5	6	7	8	9	10	11	12	Total rank[a]
1	9	9	6	9	7	8	9	9	8	9	4	6	93
2	8	8	9	8	9	7	6	6	4	1.5	6	3	75.5
3	2	2	8	7	5	9	4	8	7	8	9	9	78.0
4	6	3	4	6	4	5	8	2	6	7	7	5	63
5	4	1	2	3	1	1	3	1	3	3	1	2	25.0
6	3	4	3	1	3	2	7	5	9	6	2	7	52.0
7	6	6	7	4	6	6	5	4	1	5	5	8	63.0
9	5	7	1	2	2	3	1	7	2	1.5	3	1	35.5
10	1	5	5	5	8	4	2	3	5	4	8	8	54.0

[a] Critical values 25 and 93.

tance in obtaining the MD bacitracin premixes; and the following collaborators:

Dorothy Brennecke, Ralston Purina Co., St. Louis, MO

Gordon G. Carter, H. H. Bryant, and K. D. Jones, Food and Drug Administration, Washington, DC

Carol Harpster, A. L. Laboratories, Inc., NJ

Mary Lee Hasselberger, State Dept of Agriculture, Lincoln, NE

Linda L. Knotts, International Minerals & Chemical Corp., Terre Haute, IN

James Martin, Morris County Testing Laboratory, Convent Station, NJ

Table 10. Recovery of added bacitracin—finished feeds

	g/ton		
Sample	Theoretical	Average	Rec., %
1	9.9	4.5	45.5
2	99.5	69.9	70.3
3	488.3	326.0	66.8
4	15.7	9.6	61.4
5	158.2	120.2	76.0
6	784.3	635	80.9
7	10.0	4.6	46.3
8	99.1	73.0	73.7
9	487.4	331.0	67.9
10	16.0	11.9	74.2
11	160	125.8	78.6
12	784.5	608.2	77.5

Table 11. Analysis of variance—finished feeds study

Source of variance	Sum of sq.	DF	Mean sq.	*F*-ratio
Between laboratories	70683.42	8	8835.42	3.68
Between samples	5231499.22	11	—	—
Laboratory–sample interaction	211164.96	88	2399.60	
Total	5513347.6	107		

Jean Inglis Olsen, Hoescht-Roussel Pharmaceuticals, Somerville, NJ

H. S. Ragheb, Purdue University, West Lafayette, IN

Nancy Stouffer, State Dept of Agriculture, Boise, ID

C. Winely, Eli Lilly & Co., Indianapolis, IN

REFERENCES

(1) Gallagher, J. B., Knotts, L. L., & McCleary, D. P. (1979) 93rd Annual Meeting of the AOAC, Oct. 15–18, Washington, DC

(2) Graig, G. H. (1965) *J. Assoc. Off. Agric. Chem.* **48**, 256–264

(3) Ragheb, H. S., Black, L., & Graham, S. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 526–535

(4) Ragheb, H. S. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 444–447

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

(6) Gallagher, J. B., Love, P. W., & Knotts, L. L. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1177–1184

High Pressure Liquid Chromatographic Determination of Bacitracin in Premix Feeds and Finished Feeds: Collaborative Study

JOHN B. GALLAGHER, PAUL W. LOVE, and LINDA L. KNOTTS
International Minerals & Chemical Corp., Terre Haute, IN 47808

Collaborators: M. Allred; M. L. Hasselberger; A. Johnson; D. Louie; P. W. Love; F. Quick; D. Sharley; R. L. Smallidge; V. A. Thorpe; W. H. Williams

A liquid chromatographic technique for the determination of bacitracin in finished feeds and premix feeds consists of an isocratic reverse phase, ion-suppressed technique. The chromatography can be completed in less than 25 min. In a collaborative study involving 9 laboratories and 3 samples of bacitracin methylene disalicylic acid and 3 samples of bacitracin zinc premixes covering the range of 10-50 g/lb, the repeatability standard deviation was 0.55, and the reproducibility standard deviation was 1.35. The average recovery of the bacitracin was 102.0%. The method has been adopted official first action for bacitracin in premix feeds.

Bacitracin is a polypetide antibiotic produced by *Bacillus lichenformis* and consists of 3 active components. Bacitracin added to animal feeds for growth promotion, feed efficiency, and disease control is stabilized by reaction with zinc or methylene disalicylate (MD). It can be determined in premix feeds in the concentration range 10-50 g/lb by biological methods (1-3) or a high pressure liquid chromatographic procedure (4). The latter is a much faster and more rugged method than biological methods and provides information on component distribution.

Collaborative Study

The 3 statistical unit blocks used for this study consisted of commercial premix level feeds with nominal potencies of 10, 25, and 50 g/lb, and finished feeds at 10, 100, and 1000 g/ton levels. One member of each unit block consisted of a zinc bacitracin premix and the other a methylene disalicylate bacitracin premix.

For an intralaboratory study of the method for finished feeds, 5 lb batches of each member of each unit block were prepared by accurately weighing the commercial unmedicated premix and then adding the appropriate amount of zinc or methylene disalicylate bacitracin premix. Each batch was blended 20 min in a V-notch blender, and then ground to a consistent particle size. The materials for each unit block were riffled and packaged in moisture-proof containers. Each package was labeled with the nominal potency and a unique set of random numbers for each analyst. Five analysts were provided with a set of coded samples, 1 g zinc bacitracin standard (56.3 unit/mg), a reference chromatogram, a copy of the method, and report forms.

Bacitracin feed premixes are biomass products that do not have a known concentration of bacitracin. To evaluate method recovery, the 25 g/lb unit block was prepared in the Associate Referee's laboratory as a 1 + 1 dilution of the respective 50 g/lb premixes. A V-notch blender was used to ensure a homogeneous blend.

For the collaborative study of the method for premix feeds, materials for each unit block were riffled and packaged in moisture-proof containers. Six containers of each member of each unit block were analyzed to confirm homogeneity of the packaged samples. Each package was labeled with the nominal potency and a unique set of random numbers for each collaborator. The 11 collaborators were sent 2 samples of each member of each unit block and were asked to perform a single analysis on each of the 12 materials. Collaborators were supplied with a chromatographic column, 1 g zinc bacitracin reference standard (56.3 units/mg), a reference chromatogram, a supply of the material used to prepare the reference chromatogram, a copy of the method, and report forms. Each laboratory was also requested to submit copies of the chromatograms with the report forms.

Pretest of the Method.—In advance of the intralaboratory study and the collaborative study, the method was subjected to ruggedness testing. The following parameters were investigated: column type (4 columns), extraction time, stability of the extract, pH and organic content of

This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee G and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) **65**, 389.

Received August 21, 1981. Accepted January 8, 1982.

0004-5756/82/6505-1178-08$01.00

the chromatographic mobile phase, evaporation pH, cleanup column wash solvents, elution volumes.

Four different manufacturers' columns were tested. Three of the 4 provided satisfactory separation; however, one column provided better resolution and peak shape and is the one recommended in the procedure. Extraction time must be a minimum of 5 min and the final extract is stable 3 h at room temperature and 12 h at refrigeration temperature. The pH of the solvent system must be above 6.5 to obtain the necessary separation but pH adjustment was not a critical parameter. Normal variation in the organic content of the chromatographic mobile phase did not cause a significant variation in the results.

In addition, it has been noted during method development that HPLC columns used for other procedures would not perform satisfactorily. For this reason, the Associate Referee provided the collaborators with the chromatographic columns of various ages that have been used only for bacitracin analysis.

The cleanup column washing solvent was selected to provide maximum sample cleanup without eluting bacitracin. The cleanup column elution solvent was selected to provide complete elution of bacitracin in 3 mL solvent without eluting additional impurities.

Bacitracin in Premix Feeds
Liquid Chromatographic Method
Official First Action

42.C13 *Principle*

Bacitracin is extd from feed into acidified org. solv. system. Ext is centrfgd, and supernate is analyzed by ion-suppressed reverse phase LC with photometric detection at 254 nm.

42.C14 *Reagents and Apparatus*

(a) *Liquid chromatograph.*—Hewlett-Packard Model 1084-A, equipped with UV photometric detector. Operating conditions: flow rate 2.0 mL/min; detector wavelength 254 nm; 20 μL loop injection valve (Valco Instruments Co., Inc., Houston, TX 77055); ambient temperature.

(b) *Chromatographic column.*—15 cm × 3.0 mm id, containing 5 μm Supel Cosil LC-8 reverse phase packing (Supelco, Inc., Bellefonte, PA 16823). Use column for bacitracin analysis only.

(c) *Phosphate-EDTA buffer.*—pH 4.5. Dissolve 13.6 g KH_2PO_4 and 2.5 g EDTA in 1 L H_2O.

(d) *Phosphate buffer.*—pH 6.0. Dissolve 1.5 g K_2HPO_4 and 8.5 g KH_2PO_4 in L H_2O.

(e) *Solvent systems.*—Measure vol. indicated below with graduate (except where noted otherwise) into 100 mL vol. flask and dil. to vol. with H_2O:

	Vol. %		
Solvent	*A Solv.*	*B Solv.*	*Extg Solv.*
CH_3CN	0	40	28
MeOH	0	12	28
Phosphate-EDTA buffer	20	20	0
Phosphate buffer[a]	0	0	3
Concd phosphoric acid[a]	0	0	1.2

[a] Use vol. pipet.

(f) *Mobile phase.*—Mix 59% (v/v) B solv. with 41% (v/v) A solv. Mix, and adjust pH to 6.8 with NaOH. Slight adjustment to % vol. of B solv. may be required to obtain desired sepn.

42.C15 *Preparation of Standard*

(a) *Drying of std.—Caution:* Bacitracin is very hydroscopic. Dry std day before use and store in desiccator overnight. Accurately weigh 130–140 mg bacitracin ref. std (IMC, Terre Haute, IN 47808; 56.3 units/mg) into tared (= A) 50 mL vol. flask. Dry std 3 h at 60° under vac. at <5 mm pressure. Remove from oven and place in desiccator to cool. Reweigh (= B). Amt bacitracin std = B − A.

(b) *Preparation of std soln.—Note:* Store stds under refrigeration if not analyzed within 3 h of prepn. Preferably, prep. std, store in refrigerator >30 min before analysis, and remove from refrigerator just before analysis. *Std soln 1:* Dissolve bacitracin std in 50 mL vol. flask with ca 20 mL extg solv. and dil. to vol. Prep. following dilns from this soln. *Std soln 2:* Pipet 20 mL std soln 1 into 25 mL vol. flask; dil. to vol. with extg solv. *Std soln 3:* Pipet 15 mL std soln 1 into 25 mL vol. flask; dil. to vol. with extg solv.

42.C16 *Extraction*

Accurately weigh amt of feed contg ca 6000 units bacitracin activity into 125 mL erlenmeyer. Add 50 mL extg solv. with vol. pipet and ext with wrist-action shaking >5 min. Centrifuge 10 mL portion of ext 2–3 min at 2000–3000 rpm. Use clear supernate for assay. *Note:* Store extd sample soln under refrigeration if not analyzed within 3 h. Preferably, prep. sample solns, store

in refrigerator >30 min before analysis, and remove from refrigerator just before analysis.

42.C17 ***Determination***

Inject clear supernate from centrfgd feed and std solns into chromatograph, starting with std soln, then 2 sample solns, and then another std soln, until all samples and stds have been injected. Measure and total peak hts of the 3 active component peaks (Figure **42:C1**) for sample (PH) and std (PH') solns.

Calc. response line for stds, using least squares linear fitting of following equation:

$$PH' = m(P') + b$$

where PH' = peak hts of std solns 1, 2, and 3; P' = potency of std soln in units/50 mL for std solns 1, 2, and 3; m, b = least squares detd slope and intercept.

Det. bacitracin content of feed from:

$$\text{g bacitracin/lb} = \frac{0.01080(PH - b)}{m \times \text{sample wt}}$$

where 0.01080 = 453.6 (g/lb)/42 000 (units/g bacitracin).

Bacitracin in Finished Feeds

Principle

Feeds are washed with petroleum ether to remove fat before extraction into acidified organic solvent system. Extract is centrifuged and filtered, pH is adjusted, and organic solvent is removed using stream of air. Sample is eluted through column and analyzed by direct measurement by ion-suppressed reverse phase HPLC with photometric detector at 214 nm.

Reagents and Apparatus

(a) *Liquid chromatograph.*—See **42.C14(a)**, except wavelength 214 nm; 200 μL loop injection valve.

(b) *Goldfisch fat extractor.*—Labconco Corp., Kansas City, MO 64108.

(c) *Phosphate buffer.*—pH 4.5. Dissolve 13.6 g KH_2PO_4 in 1 L water.

(d) *Solvent systems.*—Measure volume indicated below with graduated cylinder (except where noted otherwise) into 100 mL volumetric flask and dilute to volume with water.

(e) *Preparation of standard.*—See **42.C15**, except weigh between 30 and 35 mg bacitracin reference standard.

(f) *Bond Elut columns.*—6 mL reservoir above 500 mg C-18 sorbent (Analytichem International, Inc., Harbor City, CA 90710). Prepare by washing with 5 mL eluting solvent and 5 mL solvent A.

	Volume, %				
				Cleanup Column	
Solvent	A. Solv.[a]	B. Solv.[a]	Extg solv.	Washing solv.	Eluting solv.
Acetonitrile	0	40	28	10	28
Methanol	0	12	28	3	28
Phosphate–EDTA buffer	0	0	0	20	20
pH 4.5 phosphate buffer	20	20	0	0	0
pH 6.0 phosphate buffer	0	0	3	0	0
Concd phosphoric acid[b]	0	0	1.2	0	0

[a] Adjust pH to 6.8 with concentrated NaOH.
[b] Use volumetric pipet.

Extraction

On day before assay, extract finished feeds in Goldfisch apparatus to remove fat: Accurately weigh amount of feed as specified below into folded Whatman filter paper. Place in stainless steel thimble, and extract 1 h at high temperature setting. Use 40 mL petroleum ether as solvent. Remove from Goldfisch apparatus and let air-dry overnight. On day of assay, carefully transfer treated feed to 300 mL Erlenmeyer flask.

Add 100 mL extracting solvent, using 100 mL volumetric pipet. Extract feeds ≥5 min by either shaking flask or using magnetic stirrer.

Transfer supernate to centrifuge tubes and centrifuge 10 min at 2000 rpm. Filter through glass wool into flask.

Using volumetric pipet, remove aliquot specified below and place in side-arm filtering flask. Adjust pH to 6.5 with 5N NaOH. Sparge off organic solvent by using stream of air into side-arm flask. Organic solvent is eliminated when no methanol or acetonitrile odor remains.

Transfer liquid from side-arm flask to prepared Bond Elut column and, using vacuum, draw into column. Wash flask with three 5 mL aliquots of washing solvent. Transfer each aliquot to the column and draw solution into column. All solids must be resuspended in solvent. An ultrasonic bath is useful for this purpose.

Sample weights and evaporation aliquots

Bacitracin, g/ton	Sample wt, mg	Aliquot for evapn, mL
10	50	7
50	43	2
100	43	1
250	17	1
500	8.6	1
1000	4.3	1

Table 1. Replicate results from collaborative tests reported by 9 laboratories on 6 samples of premix feeds

Lab.	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
	Replicates	Sum	Replicates	Sum	Replicates	Sum	Replicates	Sum	Replicates	Sum	Replicates	Sum
1	12.1 11.8	23.9	8.4 8.4	16.8	27.8 28.0	55.8	22.9 23.1	46.0	54.8 53.6	108.4	46.8 45.0	91.8
2	11.8 11.7	23.5	8.3 8.4	16.7	27.2 27.2	54.4	23.6 22.5	46.1	52.4 53.1	105.5	45.5 44.9	90.4
3	11.4 11.7	23.1	8.7 7.7	16.4	25.5 26.3	51.8	22.5 22.0	44.5	50.1 51.2	101.3	43.3 43.1	86.4
4	12.8 13.6	26.4	8.8 9.1	17.9	28.4 28.6	57.0	23.8 23.6	47.4	51.9 50.6	102.5	41.1 40.8	81.9
5	10.2 10.2	20.4	8.3 8.7	17.0	26.1 27.0	53.1	21.8 21.4	43.2	52.5 53.2	105.7	45.3 44.4	89.7
6	11.4 11.9	23.3	8.2 8.4	16.6	26.0 26.8	52.8	20.2 19.9	40.1	52.6 53.1	105.7	42.0 40.7	82.7
7	11.2 12.2	23.4	8.7 8.7	17.4	28.9 29.7	58.6	22.1 22.9	45.0	54.5 52.3	106.8	45.0 46.9	91.9
8	12.7 12.4	25.1	7.5 8.2	15.7	27.4 26.3	53.7	22.7 23.8	46.5	55.2 55.7	110.9	46.0 47.4	93.4
9	12.7 12.7	25.4	8.6 8.6	17.2	25.8 26.0	51.8	21.6 22.0	43.6	51.7 51.5	103.2	44.6 44.7	89.3

Elute bacitracin from Bond Elut column, using eluting solvent. Collect the first 3 mL eluate in 3 mL volumetric flask.

Samples must be stored under refrigeration if not analyzed within 3 h of extraction. For this reason, it is best to prepare samples and store in refrigerator ≥30 min before analysis, and remove from refrigerator as needed.

Determination

Inject Bond Elut column eluate and standard solutions into liquid chromatograph, and proceed as in **42.C17**.

$$\text{g bacitracin/ton} = \frac{6485.7(PH - b)}{m \times \text{evapn aliquot} \times \text{sample wt}}$$

Results and Discussion – Premix Feeds

Collaborative results were received from 9 of the 11 laboratories sent samples for analysis (Table 1). Samples 1, 3, and 5 are zinc bacitracin premixes; Samples 2, 4, and 6 are MD premixes. The collaborative study was designed so the analysis of variance procedures of both Steiner and Youden could be applied to the data (5).

The 2-sample charts for the 3 unit blocks are presented in Figure 1. As usual, the points are found predominantly in quadrants I and III, representing +,+ and −,− results: 70% of the results lie in quadrants I and III, 19% appear in quadrants II and IV, and the remaining 11% are located on the borders between quadrants. The points are generally clustered in the usual elliptical pattern around a major axis at 45°. Laboratory 3 which obtained the lowest laboratory rank is consistently located in quadrant III although the results are near the 45° line. Laboratory 6, with the most consistent error, is far removed from the 45° line in 2 of the 3 pairs, and lies just outside the circle representing the 95% confidence interval in 2 of the 3 pairs and near the circle in the remaining pair.

The chromatograms submitted by the collaborators, with one exception, were of excellent quality. The shape of the peaks indicated high chromatographic efficiency and peaks were well separated from all extraneous feed components. Collaborator 8 for some reason experienced continual downward drifting of the baseline, requiring drawing of sloping baselines; this problem did not have a serious effect on the analytical results from this laboratory (Table 1).

Rejection of Outliers.—The results of ranking the collaborative analyses are presented in Table

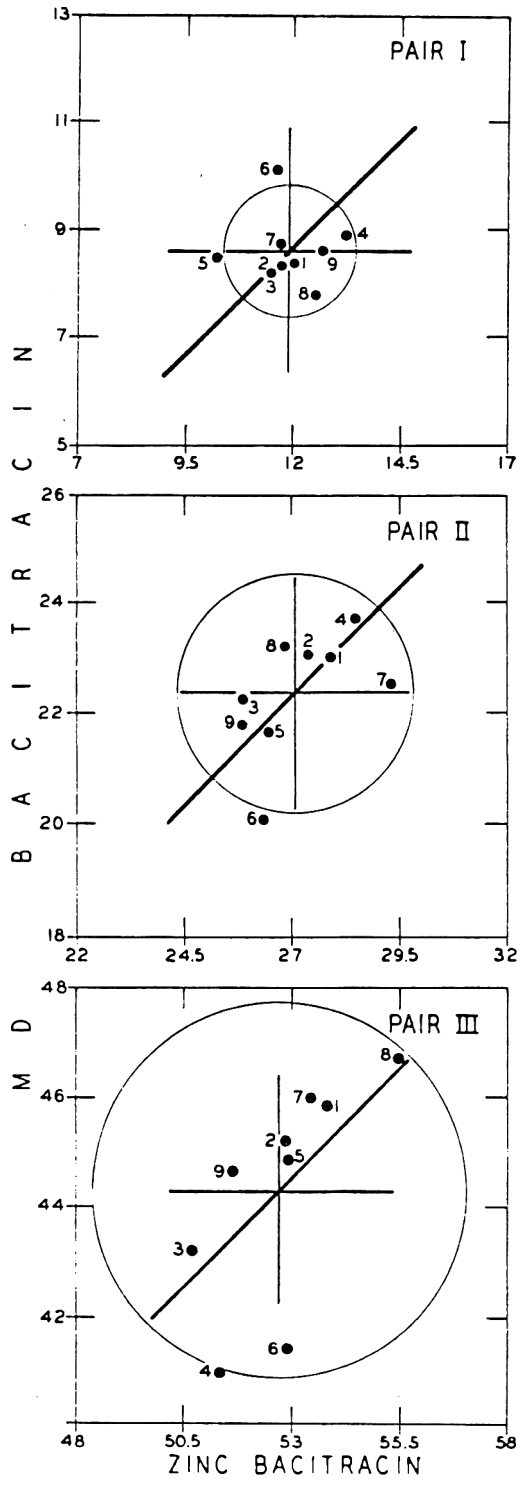


Figure 1. Two-sample charts for 3 statistical unit blocks, premix feeds. Each pair consists of a zinc and MD bacitracin premix. Numbers beside points identify collaborators. Average values are indicated by cross-hatch. Circle represents 95% confidence interval around average.

2. Only Laboratory 3 approached the approximate 5% 2-tail limits for ranking 9 collaborators with 6 samples: 13 and 47.

Table 3 presents the results of the Dixon test conducted on the sum of the duplicates to determine individual outlying results. None of the calculated values exceeded the 1 in 100 probability of 0.64 and none were rejected from the analysis. The 1 in 100 probability was selected because it provides a more cautious criterion for rejection.

Homogeneity of Variation.—The homogeneity of experimental variation between laboratories was evaluated based on the range of duplicate results on each sample. The ranges, W_s, for s = Samples 1-6 are 6.0, 4.5, 6.8, 7.3, 9.6, and 11.5 g/lb, respectively. This gives an experimental ratio W_{max}/W_{total} = 11.5/45.7 = 0.25, which is below the 1 in 20 significance level of 0.27. Therefore, there is no statistical evidence that the samples need to be handled separately.

The homogeneity of experimental variation between replicates was evaluated using Cochran's test. The experimental value for this parameter was 0.15; the critical value at the 5% significance level is 0.21. Therefore, the residual variances were regarded as homogeneous and no data were rejected.

Statistical Results.—Table 4 summarizes the results of the statistical analysis conducted by the Youden method of analysis of variance. The precision standard deviation, S_r, was computed from the difference of the average result of the paired samples, correcting S_r by $\sqrt{2}$; the distribution standard deviation, S_d, from the total of the pairs; and the standard deviation for the distribution of systematic error, S_b, from $S_d^2 = 2S_b^2 + S_r^2$. The calculated F-values in Table 3 are less than the critical F-value at the 10% level of significance for 8 degrees of freedom. Hence, the systematic error, S_b, does not make a statistically significant contribution ($P > 0.1$) to the standard deviation of the data S_d.

Table 5 summarizes the results of the statistical analysis conducted using the nested analysis of variance technique of Steiner. The between-laboratory variance ratio of 2.351 is not significant at the 10% level of significance. The laboratory-sample error is significant, which implies the method will show a greater variation when carried out between laboratories than within one

Table 2. Ranking of data from Table 1

	Sample1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6		Total
Lab.	Sum	Rank	Sum	Rank	Sum	Rank	Sum	Rank	Sum	Rank	Sum	Rank	Rank
1	23.9	6.0	16.8	5.0	55.8	7.0	46.0	6.0	108.4	8.0	91.8	7.0	39.0
2	23.5	5.0	16.7	4.0	54.4	6.0	46.1	7.0	105.5	4.0	90.4	6.0	32.0
3	23.1	2.0	16.4	2.0	51.8	1.5	44.5	4.0	101.3	1.0	86.4	3.0	13.5
4	26.4	9.0	17.9	9.0	57.0	8.0	47.4	9.0	102.5	2.0	81.9	1.0	38.0
5	20.4	1.0	17.0	6.0	53.1	4.0	43.2	2.0	105.7	5.5	89.7	5.0	23.5
6	23.3	3.0	16.6	3.0	52.8	3.0	40.1	1.0	105.7	5.5	82.7	2.0	17.5
7	23.4	4.0	17.4	8.0	58.6	9.0	45.0	5.0	106.8	7.0	91.9	8.0	41.0
8	25.1	7.0	15.7	1.0	53.7	5.0	46.5	8.0	110.9	9.0	93.4	9.0	39.0
9	25.4	8.0	17.2	7.0	51.8	1.5	43.6	3.0	103.2	3.0	89.3	4.0	26.5

Table 3. Outlying individual results for premix feeds

	Sample					
Value[a]	1	2	3	4	5	6
Highest	0.30	0.33	0.24	0.21	0.30	0.14
Lowest	0.54	0.41	0.00	0.48	0.17	0.08

[a] Critical value with 1 in 20 probability is 0.51; critical value with 1 in 100 probability is 0.64.

laboratory. The repeatability standard deviation (within-laboratory) estimate is 0.55 g bacitracin/lb. The reproducibility standard deviation (between-laboratories) estimate is 1.35 g bacitracin/lb. The laboratory-sample interaction standard deviation estimate is 1.15. These values correspond with the equivalent values S_b, S_d, and S_r obtained by the Youden procedure.

Recovery.—Because of the biomass nature of bacitracin premix products, it is only possible to obtain a method recovery. Sample Pair II was specially prepared by the Associate Referee from Sample Pair III to provide an estimation of the method recovery. Table 6 presents the results of the calculated recoveries obtained by each collaborator on both methylene disalicylate and zinc bacitracin feeds. These values were obtained by the following formula:

$$\% \text{ Recovery MD}_i = 100 \times (\text{av. value Sample 4})_i \times 2/(\text{av. value Sample 6})_i$$

$$\% \text{ Recovery zinc}_i = 100 \times (\text{av. value Sample 3})_i \times 2/(\text{av. value Sample 5})_i$$

where i represents each collaborator.

The recovery values for MD bacitracin range from 96.3 to 115.8% with an average of 101.0 ± 4.6%. Recovery values for zinc bacitracin range from 96.8 to 111.2% with an average of 103.1 ± 3.6%. By Student's *t*-test (t_{exp} = 0.834), the 2 materials have equivalent recoveries ($P > 0.1$). The overall average recovery for the method is 102.0 ± 2.64%.

Effect of Chromatographic Equipment.—The

Table 4. Results of statistical analysis for premix feeds by Youden method

	Av., g/lb							
Pair	Zinc	MD	Precision S_r	Systematic S_b	Distribution S_d	S_r^2/S_b^2	S_d^2/S_r^2	DF
I	11.92	8.62	1.078	—	0.746	—	0.48	8
II	27.17	22.35	1.124	0.597	1.406	3.54	1.56	8
III	52.73	44.30	1.516	1.190	2.265	1.62	2.23	8

Table 5. Analysis of variance for premix feeds

Source of Variance	Sum of Sq.	DF	Mean Sq.	Var. ratio
Between laboratories	0.50391E + 02	8	6.299	2.351
Between samples	0.27971E + 05	5	—	—
Lab–sample interaction	0.10716E + 03	40	2.679	8.436
Between replicates	0.17148E + 02	54	0.318	—
Total	0.28145E + 05	107		

Table 6. Percent recovery for premix feeds

	Collaborator									
Bacitracin	1	2	3	4	5	6	7	8	9	Average
MD	100.2	102.0	103.0	115.8	96.3	96.9	97.9	99.6	97.6	101.0 ± 4.6
Zinc	103.7	103.1	102.3	111.2	100.5	99.9	109.7	96.8	100.4	103.1 ± 3.6
Overall average recovery										102.0 ± 2.64

various collaborative laboratories used a variety of pumps, detectors, and injection techniques to provide the analysis for this collaborative study. Although there are not enough data to conduct a statistical analysis of the interaction of the different equipment on the analytical data, it would appear that most combinations of commercially available equipment will provide a successful analysis.

Comments of Collaborators.—One collaborator commented that his system had a dual pump capability and it would have been less difficult if the instrument had mixed the A and B solvents. We investigated this suggestion and found that if one adjusts the pH of solvents A and B and then allows the instrument to perform the solvent mixing, equivalent chromatograph performance is obtained. One collaborator had trouble obtaining the separation shown on the reference chromatogram and had to increase the flow rate to 3 mL/min. Another collaborator used a lower injection volume of 10 μL and a higher detector sensitivity than specified without adversely affecting the procedure.

Collaborator 8 indicated that weighing the sample in a volumetric flask caused a loss in precision. If the flask is brought to constant weight and the standard weight is determined by difference, this procedure would not cause a loss of precision.

Several collaborators used minor modification of the mobile phase to optimize separation.

Results and Discussion – Finished Feeds

The results of the collaborative study and statistical analysis are presented in Table 7. The precision standard deviation, S_r, was computed from the difference of the paired samples, the distribution standard deviation, S_d, from the totals of the pairs, and the standard deviation for the distribution of systematic errors, S_b, from the relationship $S_d^2 = 2S_b^2 + S_r^2$. The calculated F-values are less than the critical value at the 10% level of significance for 4 degrees of freedom. Hence, the systematic error, S_b, does not make a statistically significant contribution ($P > 0.1$) to the standard deviation of the data, S_d. The higher coefficient of variation found for the 10 g/ton samples undoubtedly relates to the higher sampling error which is associated with this material.

Recovery.—The recoveries of both zinc and methylene disalicylate bacitracin were good. The average recovery for the zinc bacitracin samples was 99.4% while the average recovery for the methylene disalicylate samples was 99.3%.

Table 7. Results of intralaboratory collaborative study of finished feeds

	Nominal potency					
	10g/ton		100g/ton		1000g/ton	
Analyst	Zinc	MD	Zinc	MD	Zinc	MD
1	9.2	9.4	110.2	117	1166	1146
2	10.1	11.3	114.0	115	1210	1140
3	9.6	10.4	118.4	122.2	1084	1124
4	9.9	9.6	113.4	121	1108	1124
5	9.7	10.7	113.0	112.4	1156	1176
Av.	9.70	10.28	113.8	117.52	1144.8	1142.0
Theor.	9.92	10.13	112.64	121.34	1151.83	1146.57
% Rec.	97.78	101.48	101.03	96.85	99.39	99.60
S_r	0.437		2.51		30.65	
S_b	0.418		2.52		22.87	
S_d	0.736		4.37		44.56	
CV, %	4.37		2.17		3.07	
S_d^2/S_r^2	2.84		3.03		2.11	

The overall recovery was 99.36% and the recoveries ranged from 96.85 to 101.48%.

Effect of Chromatographic Equipment.—Each analyst used a different column for analysis. These columns were of different ages but had been used for bacitracin analysis only. Only one detector and injector was used but 3 different pumping systems were used by analysts. Although the use of the various equipment does not generate a successful intralaboratory study, it is positive evidence of the ruggedness of the procedure.

Conclusions and Recommendations

A separate paper reports a collaborative study for premix feeds using the same unit blocks for a biological cylinder plate assay (6). The results of the 2 collaborative studies compare statistically and provide support that the HPLC procedure provides an accurate, precise, and more rapid method of analysis. Analysis of the data by the 2 statistical procedures recommended by AOAC provides added evidence of the validity of the procedure.

The Associate Referee recommends adoption of the HPLC method as official first action for premix feeds.

The method for finished feeds is very precise but still requires 2 days to complete an analysis. The chromatographic portion of the analysis can be improved by incorporation of an internal standard and use of a single calibration standard. The overall analytical time can be reduced by changing the fat removal procedure. These 2 areas are under investigation at the present, and it appears that an analysis can be performed in approximately 1 h. The Associate Referee recommends continued study.

Acknowledgments

The Associate Referee acknowledges the special efforts of L. L. Spittler for her assistance in the preparation of this manuscript; C. Harpster of A & L Laboratories for providing the MD premixes used for unit blocks I and III; and the following collaborators:

Maryann Allred, Ralston Purina Co., St. Louis, MO

Mary Lee Hasselberger, State Dept of Agriculture, Lincoln, NE

A. Johnson, International Minerals & Chemical Corp., Sterlington, LA

Dwight Louie and Floyd Quick, State Dept of Agriculture, Raleigh, NC

P. W. Love and W. H. Williams, International Minerals & Chemical Corp., Terre Haute, IN

Darla Sharley, Indiana State University, Terre Haute, IN

Robert L. Smallidge, Purdue University, West Lafayette, IN

Virginia A. Thorpe, State Dept of Agriculture, East Lansing, MI

References

(1) Craig, G. H. (1965) *J. Assoc. Off. Agric. Chem.* **48**, 256–264

(2) Ragheb, H. S., Black, L., & Graham, S. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 526–535

(3) Ragheb, H. S. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 444–447

(4) Gallagher, J. B., Knotts, L. L., & McCleary, D. P. (1979) Presented at the 93rd Annual Meeting of the AOAC, Oct. 15–18, Washington, DC

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

(6) Gallagher, J. B., & Knotts, L. J. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1167–1176

Rapid Screening Assay for Beta-Lactam Antibiotics in Milk: Collaborative Study

STANLEY E. CHARM and RUEY K. CHI[1]
New England Enzyme Center, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111

Collaborators: H. Bryant; M. Carson; W. S. Chu; A. Fraser; C. F. Gruetmacher; O. Hostetler; K. Jones; L. Kenell; R. McQuattie; C. O'Neill; D. Richwine; J. Salimbene; S. Smith; C. Wallace; R. Yokote

A 15 min assay for beta-lactam antibiotics has been used by dairies for several years as a screening procedure for testing milk tankers before they unload. The test is based on a competition between ^{14}C-penicillin and beta-lactam antibiotics in the milk samples for sites on a microbial cell wall that specifically binds beta-lactam. In a collaborative study, 11 laboratories correctly distinguished 10 coded zero penicillin G samples and 10 coded 0.01 IU/mL samples. The proposed test is qualitative, positive or negative, and can detect the presence of beta-lactam antibiotics at the 0.01 IU/mL level. The control point for determining positive or negative samples is more than 3 standard deviations from the mean of 0.01 IU/mL. The method has been adopted official first action.

Beta-lactam antibiotic (BLA) contamination of milk is a major problem in the dairy industry. There has been a need for a rapid BLA assay that can screen milk tankers before they unload at the processing plant. Up to now, methods used for detecting BLA are based on inhibition of microbial growth and require at least 2.5 h (1) which is too long for screening bulk milk shipments before unloading.

We collaboratively studied an assay that has been used for several years by dairy processors for screening milk tankers. The test requires 15 min and discriminates between zero penicillin and 0.01 IU/mL. This assay procedure, known commercially as the Charm test™ (2–4), is a qualitative test for screening fluid milk samples at antibiotic levels less than 0.01 IU/mL, based on specific, irreversible affinity of β-lactam antibiotics for certain enzyme sites on the cell wall of microorganisms (5).

In this test, ^{14}C-penicillin is the tagged BLA. An exempt quantity of carbon-14 is used so that no licensing is required on the part of the user.

Collaborative Study

A collaborative study with 12 laboratories was arranged to test the rapid BLA assay as a qualitative procedure with positives at the 0.01 IU/mL level or less. Ten of these collaborative laboratories carry out the test routinely, while 2 are government laboratories, using the test for the first time.

Coded samples, standards, and reagents were made available by Penicillin Assays Inc., Boston, MA 02111.

Penicillin in Milk

Affinity Quantitative Determination

Official First Action

(Applicable to levels ≥0.01 IU penicillin G/mL or β-lactam equiv.).

16.C01 ***Principle***

Assay is based on specific, irreversible affinity of β-lactam antibiotics for certain enzyme sites on cell wall of microorganisms. ^{14}-C-labeled penicillin and *Bacillus stearothermophilus* are added to milk sample. Antibiotic in sample competes with ^{14}C-penicillin for binding sites. Amt of bound carbon-14 is counted and compared with control to det. presence of β-lactam antibiotic.

16.C02 ***Reagents***

Note: Stds and reagents are conveniently measured into tubes, stoppered, and frozen at −20° if held >1 day. Keep reagents at <4° when used. Milk should be <15° when test starts.

(a) *Penicillin-free milk.*—Reconstitute penicillin-free whole milk powder (Yankee Milk, Newington, CT) with H_2O.

(b) *Penicillin-free skim milk powder.*—Test according to **16.131-16.136.**

This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee G and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) **65**, 389.

Received August 27, 1981. Accepted December 22, 1981.

[1] Food and Drug Administration, Division of Mathematics, Washington, DC 20204.

0004-5756/82/6505-1186-07$01.00

Table 1. Summary of correct and incorrect determinations of positive and negative coded samples of penicillin G, and standard deviations of negatives (0.00 IU/mL) and positives (0.01 IU/mL)

Lab.	Model analyzer for readout	Correct detns	Incorrect detns	Normalized mean of coded zero samples & SD (−)		Normalized mean of coded 0.01 IU/mL samples & SD (+)	
				Mean	σ zero	Mean	σ 0.01
1	150	20	0	0.93	0.06	0.52	0.03
2	300	20	0	1.07	0.10	0.57	0.05
3	300	20	0	1.02	0.06	0.59	0.05
4	300	20	0	0.94	0.11	0.59	0.10
5	150	20	0	0.93	0.04	0.51	0.03
6	150	20	0	1.05	0.11	0.59	0.07
7	150	20	0	0.96	0.08	0.55	0.06
8	300	20	0	0.95	0.06	0.62	0.03
9	300	20	0	0.96	0.06	0.52	0.03
10	150	20	0	0.96	0.09	0.56	0.04
11	150	20	0	1.05	0.09	0.65	0.09
12[a]	150	18	1[a]	0.99	0.09	0.60	0.06
Overall mean				0.98		0.57	

[a] Incorrect protocol used in this laboratory; suspect positives were not tested a second time. The incorrect positive reported here might have shown negative on the retest.

(c) *14-C Penicillin.*—Contg 103 μCi/μmole (Penicillin Assays Inc., 33 Harrison Ave, Boston, MA 02111). Use 0.0027 μCi in each assay.

(d) *B. stearothermophilus.*—Use vegetative cells, ca 47 mg wet wt in each test.

(e) *Penicillin G stds.*—*(1) Zero std:* Use penicillin-free milk, (a). *(2) 0.01 IU/mL std:* Add 1.00 IU Na or K penicillin G USP ref. std to small amt of reconstituted skim milk powder in 125 mL bottle and freeze-dry. Add 100 mL penicillin-free milk (a) and mix well.

16.C03 *Apparatus*

(a) *Heater.*—Const temp., dry well tube heater for 13 × 100 mm glass tubes (Constantemp, Roeco Manufacturing Co., Box 357, City, ST zip).

(b) *Cold plate.*—Accommodates 13 × 100 mm tubes (Penicillin Assays Inc., or equiv.).

(c) *Radiation counter.*—Models 150 or 300 mm (Penicillin Assays Inc., or equiv.).

(d) *Pipets.*—Semi-automatic (Absoluter, Tri-Continent Scientific, 12541 Loma Rica Dr, Graff Valley, CA 95945, or equiv.).

16.C04 *Procedure*

Pipet 5 mL sample into 13 × 100 mm glass tube. Pipet in 200 μL ^{14}C-penicillin and mix. Pipet in 200 μL *B. stearothermophilus* suspension and mix. Incubate 3 min in 90° dry well heater. Centrf. 4 min at 1200 × g.

Decant milk and swab out fat ring, using 2 cotton swabs. Rinse tube twice with H_2O from wash bottle; do not disturb ppt at bottom of tube. Add ca 300 μL H_2O and resuspend ppt, using tube mixer.

Place Al planchet on 400° hot plate. Pour suspension into planchet, touching mouth of tube to planchet to remove last drop. Rinse tube twice with 300 μL H_2O and add washings to planchet. Let planchet dry. Place dry planchet in penicillin analyzer and measure radiation from ^{14}C for 8 min. Compare count with predetd control point to det. whether sample is pos. or neg.

16.C05 *Control Point Determination*

Analyze 10 zero stds and average. If any zero std falls ±20% from av., replace with new std and det. new av. Control point = 0.80 × av. count. Test samples fall below control point if they contain β-lactam antibiotic or if there is a test failure.

To identify test failure, make second detn of pos. samples, zero std, and 0.01 IU/mL std at same time. Zero std count should be greater than control point, and pos. sample should again be less than control point to confirm pos. detn.

Results and Discussion

Summary of Results

Data from collaborating laboratories are summarized in Table 1. The coded sample data may be normalized by dividing with the zero standard mean for that laboratory. This facilitates comparisons between laboratories using different equipment.

The over-all normalized mean values are 0.98

Table 2. Number of standard deviation difference between control point (0.80) and coded zero means (−) and 0.01 IU/mL sample means (+), and lower limit of 95% tolerance level of zero standard mean for various laboratories ($\bar{x}_0 - 2.815\ \sigma)/\bar{x}_0$)

Lab.	No. of SD between zero mean coded sample and control point	No. of SD between 0.01 coded sample and control point	Lower limit 95% tolerance level for mean zero
1	2.20	9.30	0.78
2	2.70	4.60	0.69
3	3.67	4.20	0.92
4	1.27	5.90	0.86
5	3.25	9.70	0.80
6	2.27	3.00	0.66
7	2.00	4.17	0.81
8	2.50	6.00	0.94
9	2.70	9.30	0.66
10	1.78	6.00	0.86
11	2.79	1.67	0.75
12	2.11	3.33	0.65

for the zero samples and 0.57 for 0.01 IU/mL of penicillin G. Standard deviations range from 0.03 to 0.11 for the various laboratories.

In this assay, samples are classified as positive or negative. Those greater than 0.80 are negative and all others are positive.

A total of 239 coded samples were tested by 12 laboratories with 220 tested according to correct protocol by 11 laboratories. The laboratory not following correct protocol reported one false positive.

The 11 laboratories following correct protocol determined all samples correctly. Each laboratory had 10 positive and 10 negative samples.

Control Point

The normalized control point is 0.80 and is at least 3 standard deviations from the 0.01 IU/mL mean (except in the case of Laboratory 11). See Table 2.

It is important that this assay allows no false negatives, because there is no opportunity to recheck these, and they pass through the screen. Positives are checked a second time, and the probability of reporting a false positive is thereby reduced. The zero mean is not as far removed statistically from the control point as the 0.01 IU/mL (except in one case). The control point is selected, therefore, to pass those samples that are clearly negative (the great majority of milk samples tested) and to require a retesting of all samples that are not passed and are suspected positive.

The control point may be changed to "open" or "close" the screen. It has been found through experience that a control point of 0.8 results in a screen that does not pass false negatives and does not have an excessively high number of false positives that require a second determination.

In Table 2, a comparison is made of the control point, 0.8, and the lower limit of the 95% tolerance level from the mean of the zero standard. In some cases the 95% lower tolerance level used as a control point results in a highly variable screen compared with 0.8. The variability depends on the standard deviation associated with the laboratory.

Using the 0.8 control point, the mean of the zero concentration samples in laboratories with the greater standard deviations is less than 1.7 standard deviations from the control point (see Table 2). These laboratories have more rechecking of negatives that fall below the control point, i.e., samples appear positive on the first test but are negative on recheck.

A study by the Safeway laboratory in Landover, Maryland indicated 2 false positives in 3200 assays and no false negatives in a 6-month study (J. Reeder, Maryland and Virginia Milk Producers Association, Inc., 1981).

Proportion of Positives for Laboratories and Control

The results in Table 3 indicate that there is no significant difference in the laboratory results between the coded samples and the known control: ($X^2 = 0.08$, i.e., less than 5.04.)

Proportion of Correct Results for Negatives (0.00 IU/mL) and Positives (0.01 IU/mL)

Results in Table 4 indicate there is no significant difference between the proportion of positives and negatives correctly determined by the laboratories. The results are based on a control point that is the lower limit of the 95% tolerance level about the mean standard zero for the laboratory, i.e., $(\bar{x}_0 - 2.815\ \sigma)/\bar{x}_0$. When using this control point, 95% of the samples are detected correctly, compared with 100% when the control point is 0.80.

Agreement Between and Within Laboratories

Although there is a wide variation in the standard deviation between laboratories, each of 11 laboratories picked the same 10 positive samples and the same 10 negative samples. It is noted previously that laboratories with large standard deviations must do more work in rechecking false positive samples, but even these laboratories determined all coded positives and all coded negatives correctly.

Table 3. Comparison of coded positives determined in each laboratory with known control using lower limit of 95% tolerance level from mean of zero standard as control point [a]

	Negative Code										Positive Code										
Lab. [b]	A	B	C	E	F	J	K	N	Q	T	D	G	H	I	L	M	P	R	S	V	N_i
1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	10
2	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	10
3	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	10
4	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	12
5	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	1	1	1	10
6	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	7
7	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	10
8	1	0	1	1	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	13
9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	10
10	1	0	0	1	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	12
11	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	9
Known control	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	10
ΣN_i	3	1	1	2	0	0	0	0	0	0	12	11	11	12	12	11	11	12	12	12	123
ΣN_i^2	9	1	1	4	0	0	0	0	0	0	144	121	121	144	144	121	121	144	144	144	1363
ΣX_i	3	1	1	2	0	0	0	0	0	0	11	10	10	11	11	10	10	11	11	11	113

[a] Results transformed so that positives = 1, and negatives = 0.
[b] Lab. 12 omitted from analysis because of incorrect protocol.
N = No. of samples = 20
L = No. of labs plus known control = 12
$B = \Sigma N_i = 123$
$C = \Sigma(N_i)^2 = 1363$
$A = \Sigma X_i = 113$

$$P_1 = \frac{A}{N(L-1)} = \text{proportion of positives in laboratories} = 0.51$$

$$P_2 = \frac{B-A}{N} = \text{proportion of positives in known control} = 0.50$$

$$x^2 = \frac{[(L-1)B - LA]^2}{LB - C} = 0.08 < 5.024$$

Table 4. Comparison of correct determination of coded samples with known control using lower limit of 95% tolerance level from the mean of zero standard as control point [a]

	Negative Code										Positive Code											
Lab. [b]	A	B	C	E	F	J	K	N	Q	T	D	G	H	I	L	M	P	R	S	U	L_i	L_i^2
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20	400
2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20	400
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20	400
4	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18	324
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20	400
6	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	1	1	1	17	289
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20	400
8	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	17	289
9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20	400
10	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18	324
11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20	400
Known control	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20	400
Total	9	11	11	10	12	12	12	12	12	12	12	11	12	12	12	11	11	12	12	12	230	4426

[a] Correct results = 1; incorrect = 0.
[b] Lab. 12 omitted from analysis because of incorrect protocol.

N_1 = No. of negatives = 10
N_2 = No. of positives = 10
$N = N_1 + N_2 = 20$
L = No. of labs + known control = 12
$\Sigma L_i = T = U_1 + U_2 = 230$
$\Sigma(L_i)^2 = V = 4426$
U_1 = correct negatives = 113
U_2 = correct positives = 117

$$X^2 = \frac{(N-1)}{(N_1)(N_2)} \frac{(N_2U_1 - N_1U_2)^2}{NT - V} = \frac{19}{100} \frac{[10(113) - 10(117)]^2}{20(230) - 4426} = 1.75 < 5.04$$

$$R_1 = \text{proportion correct negatives} = \frac{U_1}{N_1 L} = \frac{113}{120} = 0.94$$

$$R_2 = \text{proportion correct positives} = \frac{U_2}{N_2 L} = \frac{117}{120} = 0.98$$

Table 5. Ruggedness test scheme, variables, and combinations of conditions

Variable		Capital letter	Lower case letter
Reagent A	Aa	freshly prepared	freeze & thaw
Reagent B	Bb	freshly prepared	freeze & thaw
Heating block	Cc	No. 1 set of 90°C	No. 2 set of 88°C
Reaction time	Dd	3 min	3 min 50 s
Centrifuge	Ee	12-tube centrifuge	6-tube centrifuge
Reagent A	Ff	most recent lot	previous lot
Reagent B	Gg	most recent lot	previous lot

(A) Sample to be tested in triplicate
(1) zero standard
(2) 0.008 standard
(B) Counter and planchet holder
Two sets of counters can be used
(1) Single unit
(2) Multi-unit
Each set of planchets had to be counted with counter 1 and counter 2.

Interference with Assay

One common interference with the assay is fat in the planchet that is read in the analyzer. The fat prevents penetration of ^{14}C-radiation and its subsequent registering by the analyzer or readout system. To avoid this, the fat ring is swabbed with 2 cotton swabs.

Reagents that deteriorate also cause false positives. Reagents, especially ^{14}C-penicillin, are sensitive to heat. Freeze-dried ^{14}C-penicillin is stable at −20°C for at least 6 months, but will deteriorate 10% in 12 h at 37°C. Reconstituted, it is stable for 12 h at less than 4°C, 24 h at −2°C, and for at least 1 month at −20°C. Reagents should not be used beyond their expiration dates. Retesting positive samples along with a zero standard checks the reagents and avoids false positives due to reagent failure.

Distribution of Measurement in Digital Readout System

Two types of digital readout systems are used with this assay. They have similar characteristics, although the numbers displayed are different. If the same planchet is measured in the same system several times, a distribution of readouts is observed whose standard deviation is approximately the square root of the average count. The reason for the distribution is that carbon-14 disintegrates in a random way. As measuring time increases, the standard deviation becomes a smaller percentage of the total count. The 8 min count is sufficient to discriminate between 0.01 IU/mL and zero.

Ruggedness Test

A ruggedness test was carried out as recommended in the AOAC Statistical Manual (6). Variables included ^{14}C-penicillin (fresh and frozen); *B. stearothermophilus* (fresh and frozen); heating block temperature; reaction time; type of centrifuge; 2 lots of reagents. The various conditions and combinations of variables tested are shown in Table 5. Statistical analysis indicated that the variables are not sensitive to the range of variation tested.

Recommendation

The results of the collaborative study indicate that the positive β-lactam samples containing 0.01 IU/mL of penicillin G are detectable and differentiated from samples containing zero β-lactam. It is recommended that the method be adopted official first action for detecting β-lactam at the 0.01 IU/mL equivalent of penicillin G or higher.

Acknowledgments

The author thanks the laboratory personnel and management of the following collaborating laboratories that took part in this study:

H. Bryant, K. Jones, and L. Kenell, Food and Drug Administration, Washington, DC

M. Carson and J. Salimbene, The Kroger Dairy Co., Indianapolis, IN

W. S. Chu, Safeway Milk Dept, Landover, MD

A. Fraser, H. P. Hood, Boston, MA

C. F. Gruetmacher, Dean Foods Co., Harvard, IL

O. Hostetler, Dairy Farm Products, Orville, OH

R. McQuattie, Borden's, Columbus, OH

C. O'Neill, Pencillin Assays, Inc., Boston, MA

D. Richwine, Dairy Farm Products, Goshen, IN

S. Smith, Foremost Foods, Dallas, TX

C. Wallace, State Dept of Agriculture, Harrisburg, PA

R. Yokote, Crystal Creamery, Sacramento, CA

The author also thanks Gordon Carter, FDA, who encouraged and guided the structure of the collaborative study; Ralston B. Read, FDA, who discussed the development of the assay; and Dairy Research, Inc., UDIA, which supported the development and commercialization of the assay.

References

(1) Ouderkirk, L. A. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 985-988

(2) Charm, S. E. (1979) *Cultured Dairy Products J.* **14,** 24-26

(3) Charm, S. E. (1980) U.S. Patent 5238521

(4) Charm, S. E. (1980) U.S. Patents 4239,745 and 4,239,852

(5) Blumberg, P. M., & Strominger, J. L. (1971) *Proc. Nat. Acad. Sci. U.S.A.* **68,** 2814-2817

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

Qualitative Ampule and Multitest for Beta-Lactam Residues in Fluid Milk Products: Collaborative Study

WESLEY N. KELLEY
State Chemical Laboratory, Vermillion, SD 57069

Collaborators: J. Andrews; A. W. Appelt; R. Barber; J. Barnett; L. Barta; B. Bass; E. Bone; L. Breske; H. H. Bryant; R. A. Case; K. Coleman; R. Cyr; S. K. Dewald; P. Dombroski; E. L. Dubs; F. F. Feldstein; B. E. Gay; R. E. Ginn; C. Gottomoller; H. Grant; J. Heady; D. G. Hills; L. Jerrod; K. Jones; C. Kaus; A. L. Lane; J. E. Leslie; D. Marchette; M. Misup; L. Morris; R. N. Mullen; C. Payton; J. Schmidt; D. Schneider; R. Share; M. Sierck; H. M. Wehr; R. Williams

A collaborative study was performed on a rapid *Bacillus stearothermophilus* agar diffusion ampule method to detect low levels of penicillin G in 7 types of fluid milk products. A multitest technique for processing a large number of samples simultaneously was also studied. Slight modifications were made in the original method to establish more uniformity and to eliminate doubtful responses by specifying a confirmation procedure. Twenty samples spiked with penicillin G (0.000 to 0.008 IU/mL) and tetracycline hydrochloride were frozen and sent to 20 laboratories in the ampule test, and 16 laboratories in the multitest. Each analyst was asked to do a screening run and a confirmation run. Results were reported by color reaction and also as positive or negative for β-lactam inhibitors. The concentrations (penicillin G) where percent positive results equal 100 or not significantly less than 100 (α = 0.05) ranged from 0.005 to 0.007 IU/mL in the ampule test and from 0.004 to 0.007 IU/mL in the multitest. Both techniques have been adopted official first action.

Since the middle of this century, a number of methods have been developed to detect the presence of antibiotic residues in milk and milk products. Most of these methods are time consuming and are not suitable for a rapid field test. In 1960, workers at Iowa State University (1) found a strain of *Bacillus stearothermophilus* suitable for detecting antibiotics in milk, using a reduction test as well as a disc assay procedure. The organism had a fast growth rate at 65°C, was sensitive to penicillin, and could be stored under refrigeration.

In 1974, a test method known as the Delvotest®-P was marketed in kit form by Gist-Brocades NV, Delft, The Netherlands. This agar diffusion test uses a strain of *B. stearothermophilus* var. *calidolactis*, and incubation in a water bath for 2½ h at 64°C. If inhibitory substances are absent, the organism produces enough acid to change the indicator from purple to yellow. If antibiotic residues are present, acid production is delayed and the agar remains purple. This has been described by Van Os et al. (2).

In 1975, Packard, Tatini, and Ginn (3) compared this test with the standard methods of the *Bacillus subtilis* disc assay and the *Sarcina lutea* cylinder plate assay. They found it to be simpler and faster than the conventional plate methods, but sensitivity was such that an excessive number of positive samples would be detected when used routinely to test farm samples. However, they felt this same sensitivity would be advantageous for testing tank trucks and storage tanks.

Pater (4) conducted a study of a large number of receiving stations in The Netherlands in 1976. He also noted the extreme sensitivity, and reported some doubtful readings.

Huhtanen et al. (5) studied this method on reconstituted dry milk powders and raw milk. They concluded that the method was "a good field technique for detecting antibiotics in raw milk and dry milk powder." They also determined that untrained analysts could detect 0.008–0.010 IU penicillin/mL 95% of the time.

In 1980, a few modifications were incorporated into the method to obtain more uniformity of results, eliminate reporting of false positives, and eliminate reporting the doubtful area of results. The modifications consisted of specifying a block heater instead of the original water bath, or baby bottle warmer, and requiring a side by side, pencillinase-treated vs heat-treated test sample confirmation before reporting results. The present study was conducted to determine if these modifications would establish a rapid, sensitive, reliable, portable method for detecting

This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee G and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) 65, 389.

Received August 21, 1981. Accepted October 22, 1981.

0004-5756/82/6505-1193-15$01.00

antibiotics in milk and milk products at the farm level as well as in receiving stations, processing plants, and regulatory laboratories.

In 1980, Van Os and Beukers (6) described the Delvotest®-P-Multitest System developed by Gist-Brocades NV. This test is similar in principle to the ampule method but permits up to 96 test cups to be used at one time. The polystyrene plate is designed so that a block of 16 cups can be broken off, depending on the number of samples to be analyzed at one time. Since the same principles apply to both the multi and ampule tests, it was felt they both should show similar results using similar modifications. However, the multitest could not be modified to use a block heater instead of a water bath; therefore it was important for each analyst to establish rigid controls to maintain the water bath temperature at a constant 65° ± 2°C for 2 h and 45 min.

Collaborative Study

A total of 20 frozen samples of 7 types of fluid milk products containing various levels of penicillin G and some containing tetracycline hydrochloride were sent to 20 and 15 laboratories to be analyzed by 30 and 26 analysts, respectively, for the ampule test and the multitest. Collaborators were divided into 2 groups (A and B). Both groups received the same number and types of samples. Only the concentration of penicillin G was varied. All analysts received at least 1 positive and 1 negative sample for each product.

Each laboratory was supplied with a block heater, liquid penicillinase, frozen standardized penicillin G (1000 IU/mL), antibiotic-free skim milk powder, a color brochure describing the test, a Delvotest-P Ampule test kit, and a Delvotest-P-Multitest kit for each analyst. Each laboratory was asked to standardize its water bath and obtain its own inhibitor-free milk for diluting standards. Instructions for conducting the test were mailed with the test samples. The experience of the analysts ranged from none to those who were running several hundred tests per week.

Raw milk was taken from a farm milk bulk tank at the completion of 3 milkings. The penicillin concentrations prepared varied from undetectable to highly detectable. One sample of each product was untreated. Two samples were spiked with 0.25 μg tetracycline hydrochloride/mL.

Processed products were selected by a 2 day survey of 3 supermarkets to determine the approximate popularity of each product; 2% lowfat milk seemed to be the most popular followed by 1% lowfat, 3.25% homogenized, skim and chocolate skim about even, and half-and-half preceding whipping cream. Because children seem to prefer chocolate products, this was selected for the "other inhibitor" sample in the processed group of samples.

Collaborators were asked to keep the samples frozen until the day of testing. Instructions were given on checking the equipment and preparing standards. Before analyzing the test samples, each analyst was asked to make a check run on 5 penicillin standards and the 3 negative controls to ensure that all reagents and equipment were satisfactory and to become familiar with the color reactions. The result of each tube or cup was recorded by color reaction on a template and interpreted as to whether further confirmation would be needed or if it was a true negative sample.

After completion of the check run, collaborators were asked to thaw the 20 test samples by partial immersion in a 50°C water bath for about 5 min, with frequent shaking, and then complete thawing at room temperature with occasional shaking. Samples were then held in the refrigerator until use, not to exceed 24 h. After use, samples were refrozen for future use. A screening run was conducted on all 20 samples simultaneously. Color reactions were recorded on a template and interpreted as "negative" or "confirmation needed." Some analysts confirmed only those samples needing confirmation, while others completed a confirmation test on all samples.

The results of 6 analysts for the ampule test, and 2 analysts for the multitest, were not included in the final tabulation because results arrived too late to be included, or the analyst could not complete the work.

Beta-Lactam in Fluid Milk Products
Qualitative Color Reaction Tests
Official First Action

16.C17 ***Principle***

Test is based on rapid growth rate and acid production of *Bacillus stearothermophilus* var. *calidolactis*. Acid changes color of bromcresol purple to yellow in absence of β-lactam inhibitors. In presence of inhibitors, purple color remains. Applicable for detecting and confirming levels of β-lactam residues ≥0.007 IU/mL processed fluid milk products and raw milk. Test

may be performed by using ampule or multitest kits.

16.C18 *Reagents and Apparatus*

(a) *Delvotest®-P-Ampule test kit.*—(GB Fermentation Industries, Inc., One N Broadway, Des Plaines, IL 60016). Contg: 100 test ampules, seeded with *B. stearothermophilus* var. *calidolactis* in plain solid agar medium (store at 4–15°); 100 nutrient tablets contg tryptone (0.5 mg), glucose (5.0 mg), nonfat dry milk (2.0 mg), and bromcresol purple (0.025 mg) (store at 4–15° until opened and then store at room temp.); plastic forceps to transfer nutrient tablets; and plastic syringe with 100 disposable tips for sampling and dispensing 0.1 mL portions of milk.

(b) *Delvotest®-P-Multi test kit.*—(GB Fermentation Industries, Inc.). Contg: hermetically sealed Al bag contg 3 plates with 96 cups each of *B. stearothermophilus* var. *calidolactis* in solid medium with bromcresol purple indicator. Each plate can be divided into 6 blocks of 16 cups each. Similar Al bag contains 3 similar plates with 1 nutrient tablet per cup, and sealing tapes to cover all blocks individually. Store at 4–15° until opened; then store at room temp.

(c) *Heaters.*—Block heater and H_2O bath, both thermostatically controlled at 65 ± 2°. Check daily.

(d) *Dispensing pipet.*—Disposable, as in ampule test, or micropipettor (Micro/Pettor, Scientific Manufacturing Industries, 1399 64th St, Emeryville, CA 94608).

(e) *Phosphate buffer.*—1%, pH 6.0. Dissolve 8.0 g anhyd. KH_2PO_4 and 2.0 g anhyd. K_2HPO_4 in H_2O and dil. to 1 L with H_2O.

(f) *Penicillinase (beta-lactamase).*—Conc., store at 0–4.4° (Difco, BBL, or Penicillin Assays, Inc., material is satisfactory).

(g) *Penicillin stock soln.*—Accurately weigh ca 30 mg USP K penicillin G ref. std and dissolve in pH 6.0 buffer to give known concn of 100–1000 IU/mL. Store at 0–4.4° not >2 days. Stock soln may be dild in inhibitor-free milk to final desired concn, distributed in small quants in tubes, sealed, and frozen ≤6 months.

(h) *Inhibitor-free milk.*—Any fluid milk product (butterfat content 0.00–3.50%, total solids <13%) may be used after being tested with this method to verify it is inhibitor-free. Use for dilg stds and as neg. control.

16.C19 *Check Run Procedure*

Perform on each new lot number of ampule or test kits and on new batches of prepd reagents.

Prep. 10 mL vols of penicillin stds in inhibitor-free milk, contg concns 0.002, 0.004, 0.006, 0.008, and 0.010 IU/mL, 10 mL H_2O, and 10 mL inhibitor-free milk in test tubes. Transfer 5 mL aliquot from each tube into a corresponding tube. Heat both sets of tubes to 82° for 3 min in H_2O bath. Remove; cool rapidly to room temp. Add 0.2 mL penicillinase to 1 series of tubes. Shake well; let stand 15 min at room temp.

For ampule test, remove and identify 1 ampule for each test tube from test kit. Break off neck of ampule and place ampule in lid of ampule box, or other suitable rack. With clean, dry forceps, place 1 nutrient tablet in each ampule. For multi test, remove 1 plate from each of the 2 foil bags. Score foil covering at cutline on end block of each plate and break off 1 block of agar cups and 1 block of cups contg nutrient tablets. Open both blocks by carefully tearing back foil. Place block contg agar upside down, exactly on top of those with nutrient tablets. Holding both blocks together, invert them and tablets will fall into each corresponding cup. Light tapping of tablet cup may be needed to get all of tablets out. Arrange test tubes of controls according to cup locations in the block. Attach dry sampling pipet to plastic syringe. Completely depress plunger, place end of pipet into tube, ca 1 cm below top of sample level. Allow plunger to return slowly under pressure of spring. Level of sample should reach wide part of pipet. If air bubble appears, slowly expel sample back into tube and let plunger slowly return again. Do not contaminate syringe. If drop of milk clings to outside of pipet tip, gently touch it off on edge of tube. This vol. is ca 0.1 mL. Empty pipet into appropriate ampule or cup. Remove pipet tip and replace with new pipet for each sample, control, or std. If using micropipettor, wipe outside with tissue and rinse 3 times in sample before removing test aliquot. Continue this pipetting procedure until all tubes have been sampled.

For ampule test, place ampules into appropriate holes in heater block. Incubate at 65 ± 2° exactly 2½ h. Remove ampules immediately. Read penicillinase-treated ampule and its corresponding untreated ampule side by side, looking through agar against white reflective background; compare and record colors as yellow, purple, or yellow-purple. Disregard intense color surrounding nutrient tablet.

For multi test, carefully seal block of cups with strip of adhesive tape enclosed in kit. Very carefully float sealed block in 65° H_2O bath. Incubate exactly 2 h and 45 min at 65 ± 2°. Remove block from H_2O bath, read, and record

colors developed. Read from bottom side of block. Compare and record colors as yellow, purple, or yellow-purple.

Following colors are satisfactory in check run: yellow for H_2O, inhibitor-free milk, and 0.002 IU/mL std; yellow or yellow-purple for 0.004 IU/mL std; purple for 0.006, 0.008, and 0.010 IU/mL stds.

Note: Occasionally kits of a particular Lot No. may require a longer incubation time for color to fully develop. If H_2O and inhibitor-free milk samples are not truly yellow and/or 0.006 and 0.008 IU/mL stds are not completely purple at end of $2\frac{1}{2}$ h incubation (2 h and 45 min for multi test) continue incubating until proper colors are developed. Check color development at 10 min intervals and record optimum incubation time required for each Lot No.

16.C20 ***Screening Procedure***

Use 1 neg. and 1 pos. control (0.008 or 0.010 IU/mL). Samples may be heat-treated, as in check run, if desired.

Prep. test ampules or blocks of cups as in check run and arrange samples in same order as ampules or cups. Shake samples 25 times through arc of 1 ft in 7 s. Using new pipet tip for each sample, control, and std, proceed with sampling and incubation as in check run. Remove ampules or blocks of cups and record colors.

All-yellow samples are neg. and need not be confirmed. Purple or yellow-purple samples must be confirmed before reporting.

16.C21 ***Confirmation Procedure***

Heat-treat two 5 mL portions of each sample to be confirmed and the 0.002, 0.004, and 0.006 IU/mL stds. Do not treat neg. control or 0.008 IU/mL std. Add 0.2 mL penicillinase to 1 portion of each heat-treated sample and the 3 low concns of stds. Prep. ampules or blocks of cups, sample, and incubate as in check run. Remove ampules or blocks and read treated and untreated samples side by side and record color results.

16.C22 ***Interpretation***

All results are reported as pos. or neg. for β-lactam residue.

Neg.: Solid yellow in screening test.

Neg.: Heated sample yellow, penicillinase-treated sample yellow.

Neg.: Heated sample purple, penicillinase-treated sample purple.

Neg.: Heated sample yellow-purple, penicillinase-treated sample yellow-purple.

Pos.: Heated sample yellow-purple, penicillinase-treated sample yellow.

Pos.: Heated sample purple, penicillinase-treated sample yellow.

Samples contg heat-stable natural inhibitor give true neg. test for β-lactam residues. Samples contg heat-stable natural inhibitor plus penicillin may result in false neg. test for β-lactam residues. Sample contg other inhibitory substances (e.g., tetracycline) will give true neg. test for β-lactam residues. Samples of chocolate-flavored products are difficult to read in the multi test kit because of light-distorting colors from adjacent cubes. They should not be reported as pos. by that method without first confirming with another method.

Results and Discussion – Ampule Test

All 24 collaborators reported negative results with the 0.002 IU/mL penicillin G standard and all negative controls (Table 1). Results of the 0.004 and 0.006 IU/mL standards were reported as 72.3% and 98.8% positive, respectively. All analysts reported the 0.008 and 0.010 IU/mL standards as positive.

Table 2 lists the results for all collaborators on the screening run made on all samples and reported as described on the screening procedure under method. All screening results listed as needing to be confirmed were confirmed and reported as either positive or negative for β-lactam residue as described in the confirmation procedure (Table 3). Table 4 summarizes the results in Tables 2 and 3 by concentration of penicillin G.

Table 4 illustrates that following the described confirmation procedure eliminates the reporting of doubtful results; all analysts reported all results as either positive or negative for β-lactam residue. Of the 168 negative results, only 4 (2.4%) needed confirmation. After confirmation, all analysts reported all samples to be negative for β-lactam residue which eliminates any problem of reporting false negative reactions.

At the 0.004 IU/mL level, 69.7% of the results indicated that confirmation was needed before reporting. After confirmation, 57.6% of the results were reported as negative and 42.4% were reported as positive. This indicates that the 0.004 IU/mL level is the approximate sensitivity of this test for fluid milk products.

All concentrations of penicillin at or above the 0.005 IU/mL level, as well as all samples spiked with tetracycline hydrochloride, were reported to need confirmation in the screening run. At the 0.005 and 0.006 IU/mL levels, 97.4% and

98.9%, respectively, of all samples were confirmed positive for β-lactam residue. At the 0.007 and 0.008 levels, all samples were confirmed as positive. All tetracycline-spiked samples were confirmed and reported as negative for β-lactam residue and all analysts reported that further testing was needed to determine the presence of other inhibitors.

The confirmed or reported results from Table 3 were statistically analyzed to (a) estimate the lowest levels (penicillin G) that have confidence limits that include 100% correct identification for each product and group (7), and (b) to compare the concentration where 50% correct identification was estimated to a similar value constructed from the current AOAC method (8).

The percent correct results for the identification of penicillin are shown in Table 5. Samples and concentrations are listed for the 2 groups of collaborators (A and B). When the same samples were analyzed by both groups, results appear in each column. Values reported as needing to be confirmed for other inhibitors were considered negative for β-lactam residues. Laboratory accidents were not considered in the analysis. The estimates of percent difference significantly less ($\alpha = 0.05$) than 100 are indicated in the tables.

Table 6 shows the lowest concentration for each product where percent positive results were not significantly less than 100%. Because concentrations did not span the entire range of all products, the level of detection could not be compared for all products. The level of detection ranged from 0.005 to 0.007 IU penicillin G/mL.

An algorithm often used to obtain estimates is based on the concentration where 50% (ED50) of analysts observed a positive result. The Spearman–Karber (9) method was used to compute the ED50 value of the controls, 0.0036 IU/mL, which is significantly less ($\alpha = 0.05$) than the 0.005 IU/mL reported for the *B. stearothermophilus* AOAC test (8).

Results and Discussion – Multitest

All 24 collaborators reported negative results for the water and negative controls and 95.8% reported their inhibitor-free milk control as negative, Table 7. Results of the 0.002 and 0.004 IU/mL penicillin G standards were reported 66.7% and 95.8% positive, respectively. All analysts reported the 0.006, 0.008, and 0.010 IU/mL penicillin standards as positive.

Table 8 lists the results for all collaborators on the screening run made on all samples and reported as described in the screening procedure under method. All screening results listed as needing confirmation were confirmed and reported as either positive or negative for β-lactam residue as described in the confirmation procedure and are reported in Table 9. Table 10 summarizes the results in Tables 8 and 9 by concentration of penicillin G.

Table 10 illustrates that following the described confirmation procedure eliminates the reporting of doubtful results because all analysts reported their results as either positive or nega-

Table 5. Ampule method summary

Sample	Concn, IU/mL	Group A($N = 11$)	Group B($N = 13$)
Raw milk	0.000	0[a]	0[a]
	0.004	36[a]	—
	0.005	—	92
	0.006	100	92
	0.008	100	92
	Tet-HCl	—	0[a]
Skim milk	0.000	0[a]	0[a]
	0.006	—	100
	0.007	100	—
1% Low fat	0.000	0[a]	0[a]
	0.004	—	55[a]
	0.005	100	—
	0.008	100	100
2% Low fat	0.000	0[a]	0[a]
	0.005	—	100
	0.006	100	—
	0.007	—	100
	0.008	100	—
Homogenized	0.000	0[a]	0[a]
	0.004	36[a]	—
	0.006	100	100
	0.007	—	100
Chocolate skim	0.000	0[a]	0[a]
	0.006	100	100
	0.008	—	100
	Tet-HCl	0[a]	—
Half-and-half	0.000	0[a]	0[a]
	0.007	100	100

[a] Significantly different from $P = 100\%$ correct identification at the $\alpha = 0.05$ level.

Table 6. Lowest β-lactam concentration where percent positive results were equal to 100 or were not significantly less than 100 at $\alpha = 0.05$ level in ampule study

Sample	Concn, IU/mL
Controls	0.006
Raw milk	0.005
Skim milk	0.006
1% Low fat	0.005
2% Low fat	0.005
Homogenized	0.006
Chocolate skim	0.006
Half-and-half	0.007[a]

[a] Lowest concentration used in study.

Table 1. Summary of results on controls used in the ampule method

Control	Screening test					Confirmed or reported results				
	Total results	Neg.	To be confirmed	% Neg.	% To be confirmed	Total results	Neg.	Pos.	% Neg.	% Pos.
Water	24	23	1	95.8	4.2	24	24	0	100.0	0.0
IFM[a]	24	24	0	100.0	0.0	24	24	0	100.0	0.0
Neg.[b] control	24	23	1	95.8	4.2	86	86	0	100.0	0.0
Penicillin stds, IU/mL:										
0.002	24	24	0	100.0	0.0	48	48	0	100.0	0.0
0.004	83	18	65	21.7	78.3	83	23	60	27.7	72.3
0.006	84	1	83	1.2	98.8	84	1	83	1.2	98.8
0.008	24	0	24	0.0	100.0	85	0	85	0.0	100.0
0.010	24	0	24	0.0	100.0	24	0	24	0.0	100.0

[a] Inhibitor-free milk, supplied by collaborator.
[b] Powdered skim milk, supplied by Associate Referee.

Table 2. Reported color results[a] of screening tests on frozen fluid milk products by ampule method

Coll.	Raw milk						Skim			1% Low fat			
	1 (0.000)[b]	6A (0.004)	6B (0.005)	10 (0.006)	13A (0.008)	13B (Tet-HCl)	9 (0.000)	2B (0.006)	2A (0.007)	3 (0.000)	11A (0.004)	11B (0.005)	16 (0.008)
1	Y	Y/P	0[c]	P	P	0	Y	0	P	Y	Y/P	0	P
2	Y	Y/P	0	Y/P	P	0	Y	0	P	Y	Y	0	P
3	Y	Y/P	0	P	P	0	Y	0	P	Y	Y/P	0	P
4	Y	Y/P	0	P	P	0	Y	0	P	Y	Y/P	0	P
5	Y	Y/P	0	P	P	0	Y	0	P	Y	Y/P	0	P
6	Y	Y/P	0	P	P	0	Y	0	P	Y	Y/P	0	P
7	Y	Y/P	0	P	P	0	Y	0	P	Y	Y/P	0	P
8	Y	Y/P	0	P	P	0	Y	0	P	Y	Y/P	0	P
9	Y	Y/P	0	Y/P	P	0	Y	0	P	Y	Y/P	0	P
10	Y	Y	0	P	P	0	Y	0	P	Y	Y/P	0	P
11	Y	Y/P	0	P	P	0	Y	0	P	Y	Y/P	0	P
% Neg.	100.0	9.1		0.0	0.0		100.0		0.0	100.0	9.1		0.0
% To be confirmed	0.0	90.9		100.0	100.0		0.0		100.0	0.0	90.9		100.0
12	Y	0	Y/P	Y/P	0	P	Y	Y/P	0	Y	0	Y/P	P
13	Y	0	Y/P	P	0	P	Y	P	0	Y	0	Y/P	P
14	Y	0	P	Y/P	0	P	Y	P	0	Y	0	P	P
15	Y	0	Y/P	P	0	P	Y	P	0	Y	0	Y/P	P

16	Y	0	P	P	0	P	Y	P	0	Y	0	Y/P	P	
17	Y	0	P	P	0	P	Y	P	0	Y	0	P	P	
18	Y	0	P	P	0	P	Y	P	0	Y	0	Y/P	P	
19	Y	0	Y/P	Y/P	0	P	Y	P	0	Y	0	Y/P	P	
20	Y	0	Y/P	P	0	P	Y	P	0	Y	0	Y/P	P	
21	Y	0	Y/P	P	0	P	Y	Y/P	0	Y	0	Y/P	LA	
22	Y	0	P	P	0	P	Y	P	0	Y	0	Y/P	P	
23	Y	0	Y/P	Y/P	0	P	Y	Y/P	0	Y/P	0	Y/P	P	
24	Y	0	Y/P	P	0	P	Y	P	0	Y	0	Y/P	P	
% Neg.	100.0		0.0	0.0		0.0	100.0	0.0		92.3		0.0	0.0	
% To be confirmed	0.0		100.0	100.0		100.0	0.0	100.0		7.7		100.0	100.0	

	2% Low fat					Homogenized				Chocolate skim				Half-and-half	
Coll.	17 (0.000)	19B (0.005)	4A (0.006)	4B (0.007)	19A (0.008)	5 (0.000)	20A (0.004)	15 (0.006)	20B (0.007)	7 (0.000)	12 (0.006)	18B (0.008)	18A (Tet-HCl)	14 (0.000)	8 (0.007)
1	Y	0	P	0	P	Y	Y/P	Y/P	0	Y	Y/P	0	0	Y/P	P
2	Y	0	Y/P	0	P	Y	Y	Y/P	0	Y	Y/P	0	0	Y	P
3	Y	0	P	0	P	Y	Y/P	P	0	Y	P	0	0	Y/P	P
4	Y	0	P	0	P	Y	Y	P	0	Y	Y/P	0	0	Y	P
5	Y	0	P	0	P	Y	Y	P	0	Y	Y/P	0	0	Y	P
6	Y	0	P	0	P	Y	Y	P	0	Y	Y/P	0	0	Y	P
7	Y	0	P	0	P	Y	Y	P	0	Y	P	0	0	Y	P
8	Y	0	P	0	P	Y	Y/P	P	0	Y	P	0	0	Y	P
9	Y	0	P	0	P	Y	Y	LA	0	Y	P	0	0	Y	P
10	Y	0	P	0	P	Y	Y	LA	0	Y	Y/P	0	0	Y	P
11	Y	0	P	0	P	Y	Y	LA	0	Y	P	0	0	Y	P
% Neg.	100.0		0.0		0.0	100.0	72.7	0.0		100.0	0.0			81.8	0.0
% To be confirmed	0.0		100.0		100.0	0.0	27.3	100.0		0.0	100.0			18.2	100.0
12	Y	Y/P	0	Y/P	0	Y	0	0	Y/P	Y	Y/P	Y/P	P	Y	P
13	Y	P	0	P	0	Y	0	0	P	Y	Y/P	P	P	Y	P
14	Y	P	0	P	0	Y	0	0	P	Y	P	P	P	Y	P
15	Y	Y/P	0	P	0	Y	0	0	P	Y	P	P	P	Y	P
16	Y	P	0	P	0	Y	0	0	P	Y	Y/P	P	P	Y	P
17	Y/P	Y/P	0	P	0	Y	0	0	P	Y	P	P	P	Y	P
18	Y	P	0	P	0	Y	0	0	P	Y	Y/P	P	P	Y	P
19	Y	Y/P	0	P	0	Y	0	0	P	Y	Y/P	P	P	Y	P
20	Y	Y/P	0	P	0	Y	0	0	P	Y	Y/P	P	P	Y	P
21	Y	Y/P	0	P	0	Y	0	0	P	Y	Y/P	P	P	Y	P
22	Y	P	0	P	0	Y	0	0	P	Y	Y/P	P	P	Y	P
23	Y	Y/P	0	Y/P	0	Y/P	0	0	Y/P	Y	Y/P	P	P	Y	P
24	Y	Y/P	0	P	0	Y	0	0	P	Y	Y/P	P	P	Y	P
% Neg.	92.3	0.0		0.0		92.3			0.0	100.0	0.0	0.0	0.0	100.0	0.0
% To be confirmed	7.7	100.0		100.0		7.7			100.0	0.0	100.0	100.0	100.0	0.0	100.0

[a] Y = Yellow, no confirmation needed; may be reported as negative. P = Purple, sample must be confirmed before reported. Y/P = Yellow–purple, sample must be confirmed before reported.

[b] Penicillin concentration, IU/mL.

[c] Sample not analyzed by this group.

Table 3. Collaborative results for analysis of frozen fluid milk samples by ampule method

	Raw milk						Skim			1% Low fat			
Coll.	1 (0.000)	6A (0.004)	6B (0.005)	10 (0.006)	13A (0.008)	13B Tet-HCl[a]	9 (0.000)	2B (0.006)	2A (0.007)	3 (0.000)	11A (0.004)	11B (0.005)	16 (0.008)
1	−	+	0[b]	+	+	0	−	0	+	−	+	0	+
2	−	+	0	+	+	0	−	0	+	−	−	0	+
3	−	+	0	+	+	0	−	0	+	−	+	0	+
4	−	−	0	+	+	0	−	0	+	−	+	0	+
5	−	−	0	+	+	0	−	0	+	−	+	0	+
6	−	−	0	+	+	0	−	0	+	−	+	0	+
7	−	−	0	+	+	0	−	0	+	−	−	0	+
8	−	−	0	+	+	0	−	0	+	−	−	0	+
9	−	−	0	+	+	0	−	0	+	−	−	0	+
10	−	−	0	+	+	0	−	0	+	−	−	0	+
11	−	+	0	+	+	0	−	0	+	−	+	0	+
Correct results, %	100	36		100	100		100		100	100	55		100
12	−	0	+	+	0	−	−	+	0	−	0	+	+
13	−	0	+	+	0	−	−	+	0	−	0	+	+
14	−	0	+	+	0	−	−	+	0	−	0	+	+
15	−	0	+	+	0	−	−	+	0	−	0	+	+
16	−	0	+	+	0	−	−	+	0	−	0	+	+
17	−	0	+	+	0	−	−	+	0	−	0	+	+
18	−	0	+	+	0	−	−	+	0	−	0	+	+
19	−	0	+	−[c]	0	−	−	+	0	−	0	+	+
20	−	0	+	+	0	−	−	+	0	−	0	+	+
21	−	0	−	+	0	−	−	+	0	−	0	+	LA
22	−	0	+	+	0	−	−	+	0	−	0	+	+
23	−	0	+	+	0	−	−	+	0	−	0	+	+
24	−	0	+	+	0	−	−	+	0	−	0	+	+
Correct results, %	100		92	92		100	100	100		100		100	100

	2% Low fat					Homogenized				Chocolate skim				Half-and-half	
Coll.	17 (0.000)	19B (0.005)	4A (0.006)	4B (0.007)	19A (0.008)	5 (0.000)	20A (0.004)	15 (0.006)	20B (0.007)	7 (0.000)	12 (0.006)	18B (0.008)	18A Tet-HCl[a]	14 (0.000)	8 (0.007)
1	−	0	+	0	+	−	+	+	0	−	+	0	−	−[c]	+
2	−	0	+	0	+	−	+	+	0	−	+	0	−	−	+
3	−	0	+	0	+	−	+	+	0	−	+	0	−	−	+
4	−	0	+	0	+	−	−	+	0	−	+	0	−	−	+
5	−	0	+	0	+	−	−	+	0	−	+	0	−	−	+
6	−	0	+	0	+	−	−	+	0	−	+	0	−	−	+
7	−	0	+	0	+	−	−	+	0	−	+	0	−	−	+
8	−	0	+	0	+	−	−	+	0	−	+	0	−	−	+
9	−	0	+	0	+	−	−	LA	0	−	+	0	−	−	+
10	−	0	+	0	+	−	+	LA	0	−	+	0	−	−	+
11	−	0	+	0	+	−	−	LA	0	−	+	0	−	−	+

Correct results, %	100		100		100	100	36	100		100	100		100	100	100	
12	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
13	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
14	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
15	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
16	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
17	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
18	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
19	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
20	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
21	–	LA	0	+	0	–	0	+	+	–	+	LA	0	–	+	
22	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
23	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
24	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+	
Correct results, %	100	100		100		100		100	100	100	100	100		100	100	

[a] Reported as negative for β-lactam inhibitor, all 11 analysts also reported that additional testing was needed to confirm that another inhibitor was present.
[b] Sample not analyzed by this group.
[c] Incorrectly reported as other inhibitor.

Table 4. Summary of collaborative results reported by penicillin concentration on frozen fluid milk products by ampule method

	Screening test					Confirmed or reported results				
Pencillin concn, IU/mL	Total results	Neg.	To be confirmed	% Neg.	% To be confirmed	Total results	Neg.	Pos.	% Neg.	% Pos.
0.000	168	164	4	97.6	2.4	167[a]	167	0	100.0	0.0
0.004	33	10	23	30.3	69.7	33	19	14	57.6	42.4
0.005	39	0	39	0.0	100.0	38[b]	1	37	2.6	97.4
0.006	93	0	93	0.0	100.0	93[a,c]	1	92	1.1	98.9
0.007	61	0	61	0.0	100.0	60	0	60	0.0	100.0
0.008	59	0	59	0.0	100.0	57[d]	0	57	0.0	100.0
Tet-HCl	24	0	24	0.0	100.0	24	24[e]	0	100.0	0.0

[a] 1 incorrectly reported as other inhibitor.
[b] 1 laboratory accident.
[c] 3 laboratory accidents.
[d] 2 laboratory accidents.
[e] All collaborators reported further testing needed to confirm other inhibitors.

Table 7. Summary of results on controls used in multitest method

Control	Screening test					Confirmed or reported results				
	Total results	Neg.	To be confirmed	% Neg.	% To be confirmed	Total results	Neg.	Pos.	% Neg.	% Pos.
Water	24	24	0	100.0	0.0	24	24	0	100.0	0.0
IFM[a]	72	66	6	91.7	8.3	24	23	1	95.8	4.2
Neg.[b] control	72	70	2	97.2	2.8	24	24	0	100.0	0.0
Pen. stds, IU/mL:										
0.002	—	—	—	—	—	24	8	16	33.3	66.7
0.004	48	2	46	4.2	95.8	24	1	23	4.2	95.8
0.006	72	1	71	1.4	98.6	24	0	24	0.0	100.0
0.008	72	0	72	0.0	100.0	48	0	48	0.0	100.0
0.010	72	0	72	0.0	100.0	—	—	—	—	—

[a] Inhibitor-free milk, supplied by collaborator.
[b] Powdered skim milk, supplied by Associate Referee.

Table 8. Reported color results[a] of screening tests on frozen fluid milk products by multitest method

Coll.	Raw milk						Skim			1% Low fat			
	1 (0.000)	6A (0.004)	6B (0.005)	10 (0.006)	13A (0.008)	13B Tet-HCl	9 (0.000)	2B (0.006)	2A (0.007)	3 (0.000)	11A (0.004)	11B (0.005)	16 (0.008)
1	Y/P	Y/P	0[b]	Y/P	P	0	Y	0	P	Y/P	Y/P	0	P
2	Y	P	0	P	P	0	Y	0	P	Y	P	0	P
3	Y	P	0	P	P	0	Y	0	P	Y	Y/P	0	P
4	Y	Y/P	0	P	P	0	Y	0	P	Y	P	0	P
5	Y	P	0	P	P	0	Y	0	P	Y	P	0	P
6	Y	P	0	P	P	0	Y	0	P	Y	P	0	P
7	Y	P	0	P	P	0	Y	0	P	Y	P	0	P
8	Y	P	0	P	P	0	Y	0	P	Y	Y/P	0	P
9	Y	P	0	P	P	0	Y	0	P	Y	Y/P	0	P
10	Y	P	0	P	P	0	Y	0	P	Y	Y/P	0	P
11	Y	Y/P	0	P	P	0	Y	0	P	Y	Y/P	0	P
12	Y	Y/P	0	P	P	0	Y	0	P	Y	P	0	P
13	Y	Y/P	0	P	P	0	Y	0	P	Y	Y/P	0	P
% Neg.	92.3	0.0		0.0	0.0		100.0		0.0	92.3	0.0		0.0
% To be confirmed	7.7	100.0		100.0	100.0		0.0		100.0	7.7	100.0		100.0
14	Y	0	Y/P	P	0	P	Y	P	0	Y	0	Y/P	P
15	Y	0	P	P	0	P	Y	P	0	Y	0	Y/P	P
16	Y	0	P	P	0	P	Y	P	0	Y	0	P	P
17	Y	0	P	P	0	P	Y	P	0	Y	0	P	P

18	Y	0	P	P	0	P	Y	P	0	Y	0	P	P
19	Y	0	P	P	0	P	Y	P	0	Y	0	Y/P	P
20	Y	0	P	P	0	P	Y	P	0	Y	0	P	P
21	Y	0	P	P	0	P	Y	P	0	Y	0	P	P
22	Y	0	P	P	0	P	Y	P	0	Y	0	P	P
23	Y	0	P	P	0	P	Y	P	0	Y	0	P	P
24	Y	0	P	P	0	P	Y	P	0	Y	0	P	P
% Neg.	100.0		0.0	0.0		0.0	100.0	0.0		100.0		0.0	0.0
% To be confirmed	0.0		100.0	100.0		100.0	0.0	100.0		0.0		100.0	100.0

	2% Low fat					Homogenized				Chocolate skim				Half-and-half	
Coll.	17 (0.000)	19B (0.005)	4A (0.006)	4B (0.007)	19A (0.008)	5 (0.000)	20A (0.004)	15 (0.006)	20B (0.007)	7 (0.000)	12 (0.006)	18B (0.008)	18A (Tet-HCl)	14 (0.000)	8 (0.007)
1	Y	0	P	0	P	Y	Y/P	P	0	Y	P	0	P	Y	P
2	Y	0	P	0	P	Y	Y/P	P	0	Y	P	0	P	Y	P
3	Y	0	P	0	P	Y	Y/P	P	0	Y	P	0	P	Y	P
4	Y/P	0	P	0	P	Y	P	P	0	Y/P	P	0	P	Y	P
5	Y/P	0	P	0	P	Y/P	P	P	0	Y/P	P	0	P	Y	P
6	Y	0	P	0	P	Y/P	Y/P	P	0	Y	P	0	P	Y	Y/P
7	Y	0	P	0	P	Y	P	P	0	Y	P	0	P	Y	P
8	Y	0	P	0	P	Y	Y/P	P	0	Y/P	P	0	P	Y	P
9	Y	0	P	0	P	Y	Y/P	P	0	Y/P	P	0	P	Y	P
10	Y	0	P	0	P	Y	Y/P	P	0	Y/P	P	0	P	Y	P
11	Y	0	P	0	P	Y	Y/P	P	0	Y/P	P	0	P	Y	P
12	Y	0	P	0	P	Y	Y/P	P	0	Y	P	0	P	Y	P
13	Y	0	P	0	P	Y	Y/P	P	0	Y	P	0	P	Y	P
% Neg	84.6		0.0		0.0	84.6	0.0	0.0		46.2	0.0		0.0	100.0	0.0
% To be confirmed	15.4		100.0		100.0	15.4	100.0	100.0		53.8	100.0		100.0	0.0	100.0
14	Y	P	0	P	0	Y	0	P	P	Y/P	P	P	0	Y	P
15	Y	P	0	P	0	Y	0	P	P	Y/P	P	P	0	Y	P
16	Y	P	0	P	0	Y	0	P	P	Y/P	P	P	0	Y	P
17	Y	P	0	P	0	Y	0	P	P	Y	P	P	0	Y	P
18	Y	P	0	P	0	Y	0	P	P	Y	P	P	0	Y	P
19	Y	P	0	P	0	Y	0	P	P	Y	P	P	0	Y	P
20	Y	P	0	P	0	Y	0	P	P	Y	P	P	0	Y	P
21	Y	P	0	P	0	Y	0	P	P	Y/P	P	P	0	Y	P
22	Y	P	0	P	0	Y	0	P	P	Y	P	P	0	Y	P
23	Y	P	0	P	0	Y	0	P	P	Y/P	P	P	0	Y	P
24	Y/P	P	0	P	0	Y	0	P	P	Y	P	P	0	Y	P
% Neg.	91.0	0.0		0.0		100.0		0.0	0.0	54.5	0.0	0.0		100.0	0.0
% To be confirmed	9.0	100.0		100.0		0.0		100.0	100.0	45.5	100.0	100.0		0.0	100.0

[a] Y = Yellow, no confirmation needed; may be reported as negative. P = Purple, sample must be confirmed before reported. Y/P = Yellow–purple, sample must be confirmed before reported.

[b] Sample not analyzed by this group.

Table 9. Collaborative results of analysis of frozen fluid milk samples by multitest method

	Raw milk						Skim			1% Low fat			
Coll.	1 (0.000)	6A (0.004)	6B (0.005)	10 (0.006)	13A (0.008)	13B (Tet-HCl)[a]	9 (0.000)	2B (0.006)	2A (0.007)	3 (0.000)	11A (0.004)	11B (0.005)	16 (0.008)
1	−	+	0[b]	+	+	0	−	0	+	−	+	0	+
2	−	+	0	+	+	0	−	0	+	−	+	0	+
3	−	+	0	+	+	0	−	0	+	−	+	0	+
4	−	+	0	+	+	0	−	0	+	−	+	0	+
5	−	+	0	+	+	0	−	0	+	−	+	0	+
6	−	+	0	+	+	0	−	0	+	−	+	0	+
7	−	+	0	+	+	0	−	0	+	−	+	0	+
8	−	+	0	+	+	0	−	0	+	−	+	0	+
9	−	+	0	+	+	0	−	0	+	−	+	0	+
10	−	+	0	+	+	0	−	0	+	−	+	0	+
11	−	+	0	+	+	0	−	0	+	−	+	0	+
12	−	−	0	+	+	0	−	0	+	−	+	0	+
13	−	+	0	+	+	0	−	0	+	−	+	0	+
Correct results, %	100	92		100	100		100		100	100	100		100
14	−	0	+	+	0	−	−	+	0	−	0	+	+
15	−	0	+	+	0	−	−	+	0	−	0	+	+
16	−	0	−[c]	+	0	−	−	+	0	−	0	+	+
17	−	0	+	+	0	−	−	+	0	−	0	+	+
18	−	0	+	+	0	−	−	+	0	−	0	+	+
19	−	0	+	+	0	−	−	+	0	−	0	+	+
20	−	0	+	+	0	−	−	+	0	−	0	+	+
21	−	0	+	+	0	−	−	+	0	−	0	+	+
22	−	0	+	+	0	−	−	+	0	−	0	+	+
23	−	0	+	+	0	−	−	+	0	−	0	+	+
24	−	0	+	+	0	−	−	+	0	−	0	+	+
Correct results, %	100		91	100		100	100	100		100		100	100

	2% Low fat					Homogenized				Chocolate skim				Half-and-half	
Coll.	17 (0.000)	19B (0.005)	4A (0.006)	4B (0.007)	19A (0.008)	5 (0.000)	20A (0.004)	15 (0.006)	20B (0.007)	7 (0.000)	12 (0.006)	18B (0.008)	18A (Tet-HCl)	14 (0.000)	8 (0.007)
1	−	0	+	0	+	−	+	+	0	−	+	0	−	−	+
2	−	0	+	0	+	−	+	+	0	−	+	0	−	−	+
3	−	0	+	0	+	−	+	+	0	−	+	0	−	−	+
4	−	0	+	0	+	−	+	+	0	−[c]	−[c]	0	−	−	+
5	−	0	+	0	−[c]	−	+	+	0	−[c]	−[c]	0	−	−	+
6	−	0	+	0	+	−	+	+	0	−	+	0	−	−	+
7	−	0	+	0	+	−	+	+	0	−	+	0	−	−	+
8	−	0	+	0	+	−	+	+	0	−	−[c]	0	−	−	+
9	−	0	+	0	+	−	+	+	0	−	−[c]	0	−	−	+
10	—	0	+	0	+	−	+	+	0	−	−[c]	0	−	−	+
11	−	0	+	0	+	−	+	+	0	−	−[c]	0	−	−	+
12	−	0	+	0	+	−	−[c]	+	0	−	+	0	−	−	+
13	−	0	+	0	+	−	+	+	0	−	+	0	−	−	+

Correct results, %	100		100		92	100	92	100		100	45		100	100	100
14	–	+	0	+	0	–	0	+	+	–[c]	–[c]	–[c]	0	–	+
15	–	+	0	+	0	–	0	+	+	–[c]	–[c]	–[c]	0	–	+
16	–	+	0	–[c]	0	–	0	+	+	–[c]	–[c]	–[c]	0	–	+
17	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+
18	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+
19	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+
20	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+
21	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+
22	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+
23	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+
24	–	+	0	+	0	–	0	+	+	–	+	+	0	–	+
Correct results, %	100	100		91		100		100	100	100	77	73		100	100

[a] Reported as negative for β-lactam inhibitor, all analysts also reported that additional testing was needed to confirm the presence of another inhibitor.
[b] Sample not analyzed by this group.
[c] Incorrectly reported as other inhibitor.

Table 10. Summary of collaborative results reported by penicillin concentration on frozen fluid milk products by multitest

	Screening test					Confirmed or reported results				
Penicillin concn, IU/mL	Total results	Neg.	To be confirmed	% Neg.	% To be confirmed	Total results	Neg.	Pos.	% Neg.	% Pos.
0.000	168	150	18	89.3	10.7	168	168[a]	0	100.0	0.0
0.004	39	0	39	0.0	100.0	39	2[b]	37	5.1	94.9
0.005	33	0	33	0.0	100.0	33	1[b]	32	3.0	97.0
0.006	96	0	96	0.0	100.0	96	9[c]	87	9.4	90.6(100.0)[d]
0.007	59	0	59	0.0	100.0	59	1[b]	58	1.7	98.3
0.008	61	0	61	0.0	100.0	61	4[b,e]	57	6.6	93.4(97.9)[d]
Tet-HCl	24	0	24	0.0	100.0	24	24[f]	0	100.0	0.0

[a] 5 analysts reported confirmation testing needed for other inhibitors.
[b] 1 analyst reported confirmation testing needed for other inhibitors.
[c] All analysts reported confirmation testing needed for other inhibitors in chocolate products.
[d] Revised after removing results for chocolate skim milk from data.
[e] 3 analysts reported confirmation testing needed for other inhibitors in chocolate products.
[f] All analysts reported confirmation testing needed for other inhibitors in all samples.

Table 11. Multitest summary

Sample	Concn, IU/mL	Group A(N = 11)	Group B(N = 13)
Raw milk	0.000	0[a]	0[a]
	0.004	92	—
	0.005	—	91
	0.006	100	100
	0.008	100	—
	Tet-HCl	—	0[a]
Skim milk	0.000	0[a]	0[a]
	0.006	—	100
	0.007	100	—
1% Low fat	0.000	0[a]	0[a]
	0.004	100	—
	0.005	—	100
	0.008	100	100
2% Low fat	0.000	0[a]	0[a]
	0.005	—	100
	0.006	100	—
	0.007	—	91
	0.008	92	—
Homogenized	0.000	0[a]	0[a]
	0.004	92	—
	0.006	100	100
	0.007	—	100
Chocolate skim	0.000	0[a]	0[a]
	0.006	45[a]	77[a]
	0.008	—	73[a]
	Tet-HCl	0[a]	—
Half-and-half	0.000	0[a]	0[a]
	0.007	100	100

[a] Significantly different from P = 100% correct identification at the α = 0.05 level.

tive for β-lactam residues. Of the 168 negative results, 18 (10.7%) were determined to need confirmation. After confirmation, all analysts reported all samples to be negative for β-lactam residue.

All samples spiked with either penicillin or tetracycline hydrochloride were reported to need confirmation after the screening test. All samples spiked with tetracycline were correctly reported as negative for β-lactam residue and as needing additional testing for other inhibitors. Table 10 shows that 90.6–98.3% of all results were confirmed as β-lactam residues for all samples spiked with penicillin. Several analysts reported difficulty in reading the color reaction with the chocolate skim milk samples. This is illustrated in Tables 8 and 9. Most collaborators felt that the color diffusion from adjacent sample cups, along with the natural color of the chocolate material, caused the problem. If the results of the chocolate samples are removed from the calculations in Table 10, we find that the percentage of correctly reported positive results increases to 94.9% at the 0.003 level and to 100.0% at the 0.006 IU/mL level.

Table 12. Lowest β-lactam concentration where percent positive results were equal to 100 or were not significantly less than 100 at the α = 0.05 level in the multitest study

Sample	Concn, IU/mL
Controls	0.004
Raw milk	0.004
Skim milk	0.006
1% Low fat	0.004
2% Low fat	0.005
Homogenized	0.004
Chocolate skim	—[a]
Half-and-half	0.007[b]

[a] None determined due to numerous incorrect other inhibitors reported.

[b] Lowest concentration used in study.

The confirmed or reported results from Table 9 were statistically analyzed (a) to estimate the lowest levels (penicillin G) that have confidence limits that include 100% correct identification for each product and group (7), and (b) to compare the concentration where 50% correct identification was estimated to a similar value constructed from the current AOAC method (8).

The percent correct results for the identification of penicillin are shown in Table 11. Samples and concentrations are listed for the 2 groups of collaborators (A and B). When the same samples were analyzed by both groups, results appear in each column. Values reported as needing to be confirmed for other inhibitors were considered negative for β-lactam residues. Laboratory accidents were not considered in the analysis. The estimates of percent differences significantly less (α = 0.05) than 100 are indicated in Table 11.

Table 12 shows the lowest concentration for each product where percent positive results were significantly less than 100%. This concentration could not be determined for chocolate skim milk with this method. All negative results in Table 11 (chocolate skim milk) were due to incorrectly reported negatives needing further confirmation for other inhibitors. The level of detection for all other samples ranged from 0.004 to 0.007 IU penicillin G/mL.

The Spearman-Karber (9) method was used to compute the ED50 value of the controls, 0.0018 IU/mL, which is significantly less (α = 0.05) than the 0.005 IU/mL reported for the *B. stearothermophilus* AOAC test (8).

Recommendation

A rapid, reliable, sensitive, and portable method for detecting antibiotics in large num-

bers of milk samples at the farm and receiving station levels is needed. The results of this collaborative study show that the Delvotest®-P-Multitest and Delvotest®-P-Ampule method can fulfill these requirements on raw milk and on processed fluid milk products. It is recommended that, except for the multitest on chocolate-flavored milk products, both techniques be adopted official first action for the detection of β-lactam residues in milk and milk products.

Acknowledgments

I wish to thank J. G. Dooley and G. B. Fermentation Industries, Inc., Des Plaines, IL, for supplying the equipment and reagents, and Ray Hall Dairy, Burbank, SD, for supplying the raw milk samples used in this study. A special thanks to J. W. Messer, Food and Drug Administration, for his technical advice and J. T. Peeler, Food and Drug Administration, for his statistical analysis of the results. I am also deeply indebted to the following collaborators without whom this study would not have been possible:

J. Andrews and B. Bass, Land O'Lakes Dairy, Lake Norden, SD

A. W. Appelt and S. K. Dewald, State Chemical Laboratory, Vermillion, SD

R. Barber, F. F. Feldstein, and C. Gottomoller, ESS Laboratory Services, Ltd, College Park, MD

J. Barnett and J. E. Leslie, Food and Drug Administration, Cincinnati, OH

L. Barta and R. E. Ginn, Dairy Quality Control Institute, Inc., St. Paul, MN

E. Bone, P. Dombroski, B. E. Gay, and C. Payton, State Dept of Public Health, Springfield, IL

L. Breske, L. Jerrod, and M. Sierck, Terrace Park Dairy, Sioux Falls, SD

H. H. Bryant and K. Jones, Food and Drug Administration, Washington, DC

R. A. Case and R. Share, Kraft Inc., Chicago, IL

K. Coleman, Dairymen, Inc., Spartanburg, SC

R. Cyr and R. N. Mullen, State Dept of Agriculture, Montpelier, VT

E. L. Dubs, C. Kaus, and J. Schmidt, AMPI Dairy, Freeman, SD

H. Grant and D. Marchette, State Dept of Agriculture, Winter Haven, FL

J. Heady, Missouri Division of Health, Jefferson City, MO

D. G. Hills, Kraft Inc., Milledgeville, IL

A. L. Lane and L. Morris, Difco Laboratories, Detroit, MI

M. Misup, State Dept of Public Health, Chicago, IL

D. Schneider, Kraft Inc., Beaver Dam, WI

H. M. Wehr and R. Williams, State Dept of Agriculture, Salem, OR

References

(1) Igarashi, R. T., Baughman, R. W., Nelson, F. E., & Hartman, P. A. (1961) *J. Milk Food Technol.* **24,** 143–146

(2) Van Os, J. L., Lameris, S. A., Doodewaard, T., & Oostendorp, J. G. (1975) *Neth. Milk Dairy J.* **29,** 16–34

(3) Packard, V. S., Tatini, S., & Ginn, R. E. (1975) *J. Milk Food Technol.* **38,** 601–603

(4) Pater, B. A. (1977) *J. Food Prot.* **40,** 23–24

(5) Huhtanen, C. N., et al. (1977) *J. Food Prot.* **40,** 236–240

(6) Van Os, J. L., & Beukers, R. (1980) *J. Food Prot.* **43,** 510–511

(7) Pearson, E. S., & Hartley, H. O. (1970) *Biometrika Tables for Statisticians,* Vol. 1, 3rd Ed., Cambridge University Press, Cambridge, UK, pp. 83–84, 228

(8) Ouderkirk, L. A. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 985–988

(9) Finney, D. J. (1978) *Statistical Methods in Biological Assay,* Macmillan Publishing Co., New York, NY

Bacillus stearothermophilus Disc Assay for Detection of Inhibitors in Milk: Collaborative Study

JAMES W. MESSER, JAMES E. LESLIE, GARY A. HOUGHTBY, JAMES T. PEELER, and JERALD E. BARNETT
Food and Drug Administration, Division of Microbiology, 1090 Tusculum Ave, Cincinnati, OH 45226

Collaborators: A. Appelt; E. Boone; H. Bryant; L. Burroughs; K. Coleman; M. Cupery; R. Cyr; P. Dombrowski; P. Donahue; S. Farmer; R. Ginn; H. Grant, Jr; J. Hawkins; J. Heady; D. Herbst; W. Kelley; M. Misup; L. Morris; R. Mullen; N. Ondrus; C. Payton; D. Seiz; R. Share; M. Stern; R. Williams

A 2-part (A and B) collaborative study was conducted on a *Bacillus stearothermophilus* paper disc (12.7 mm) method to detect residual inhibitors in milk. The 18 participating collaborators assayed raw milk samples spiked with a beta-lactam (penicillin G). Of the 18 collaborators, 14 participated in part A and 16 in part B. Part A demonstrated that either Antibiotic Medium No. 4 or PM Indicator Agar is suitable for use in the assay. The lowest concentration detectable, not significantly different from 100% at the $\alpha = 0.05$ level, was 0.008 unit/mL with either medium. Part B demonstrated that the sensitivity of the method is equal to that of the current AOAC method (16.131-16.136). The concentration of beta-lactam detected by 50% of the analysts was 0.003-0.005 unit/mL in this study, compared with 0.005 unit/mL reported in an earlier collaborative study on the current AOAC method. No false positive results were reported in part A or part B. All samples found positive by the confirmatory test in part B were correctly identified as a beta-lactam with commercial Penase discs. The lowest concentration detectable by the method, not significantly different from 100% at the $\alpha = 0.05$ level, was 0.008 unit/mL. The method was adopted official first action.

Since the late 1940s, antibiotics and drugs have been widely used to control disease in dairy cattle. Milk from treated animals that is not withheld from the milk supply for the time required for excretion of the antibiotic or drug may contain residual levels of inhibitors and may not be sold (1). Several microbiological methods are officially recognized to detect residual levels of inhibitors (antibiotics and/or drugs) in milk (2, 3). The method presented was designed as an alternative equivalent to the official AOAC method **16.131-16.136** (3).

Received August 6, 1981. Accepted October 26, 1981.
This report of the Associate Referee, J. W. Messer, was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.
The recommendation of the Associate Referee was approved by the General Referee and Committee G and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) **65**, 389.

Collaborative Study

Ten frozen raw milk samples were prepared with milk that had been previously tested for the presence of inhibitors by the *Bacillus stearothermophilus* method. A buffered stock solution of penicillin G was prepared (1000 units/mL) and diluted in the inhibitor-free milk to give 4 spiked samples. One additional sample was used as a negative control. The 5 samples were subdivided to provide a blind duplicate. The 10 samples were packaged in 40 mL amounts in sterile polystyrene bottles, divided into replicate sets of 10 samples, and frozen.

The collaborative study was carried out in 2 parts. Part A was designed to familiarize the collaborators with the method and to determine whether PM Indicator Agar would produce assay results similar to those obtained with Antibiotic Medium No. 4. Part B was designed to study the use of the method at 2 incubation temperatures and to determine whether commercial Penase discs could be used to identify the residue as a beta-lactam.

For each part of the study, 10 frozen samples, essential materials, and instructions were provided to each collaborator. The identities of the samples and blind duplicates were unknown to the participants. The collaborators sent their data forms to the Associate Referee who decoded the data and had the statistical analysis (4-6) performed. For purposes of comparison, the 50% detectable level (ED50) was used (5).

Penicillins in Milk

Bacillus stearothermophilus Qualitative Disc Method II

Official First Action

(Applicable to levels ≥0.008 IU penicillin G/mL)

Table 1. Beta-lactam residues in heated milk detected by using Antibiotic Medium No. 4, incubated at 55 ± 2°C

	Penicillin G (unit/mL)									
	0.000/mL sample		0.002/mL sample		0.004/mL sample		0.008/mL sample		0.012/mL sample	
Coll.	1	9	2	5	7	8	3	4	6	10
1	–[a]	–	–	–	+[b]	–	+	+	+	+
2	–	–	–	–	+	+	+	+	+	+
3	–	–	–	–	+	–	+	+	+	+
4	–	–	–	–	+	+	+	+	+	+
5	–	–	–	–	+	+	+	+	+	+
6	–	–	–	–	–	–	+	+	+	+
7	–	–	–	–	+	+	+	+	+	+
10	–	–	–	–	+	+	+	+	+	+
11	–	–	–	–	–	+	+	+	+	+
12	–	–	–	–	–	–	+	+	+	+
13	–	–	–	–	–	–	+	+	+	+
14	–	–	–	–	+	+	+	+	+	+
15	–	–	–	–	–	–	+	+	+	+
18	–	–	–	–	–	–	+	+	+	+
Correct results (%)	100		0[c]		54[c]		100		100	

[a] – = negative test (zone ≤ 14.0 mm).
[b] + = positive test (zone > 14.0 mm).
[c] Significantly different from 100% at the $\alpha = 0.05$ level.

16.C06 ***Culture Media***

(a) *Agar medium B.*—See **42.196(b)**.

(b) *Agar medium P.*—Dissolve 3.0 g beef ext, 5.0 g peptone, 1.7 g tryptone, 0.3 g soytone, 5.25 g dextrose, 0.5 g NaCl, 0.25 g K_2HPO_4, 1.0 g polysorbate 80, 0.06 g bromcresol purple, and 15.0 g agar in H_2O and dil. to 1 L. Adjust if necessary so that after sterilization pH is 7.8 ± 0.2. (Difco PM Indicator Agar has been found satisfactory.)

(c) *Agar medium M.*—See **16.131(b)**.

Table 2. Beta-lactam residues in heated milk detected by using PM Indicator Agar[a] incubated at 55 ± 2°C

	Penicillin G (unit/mL)									
	0.000/mL sample		0.002/mL sample		0.004/mL sample		0.008/mL sample		0.012/mL sample	
Coll.	1	9	2	5	7	8	3	4	6	10
1	–[b]	–	–	–	+[c]	+	+	+	+	+
2	–	–	–	–	+	+	+	+	+	+
3	–	–	+	+	+	+	+	+	+	+
4	–	–	–	–	+	+	+	+	+	–
5	–	–	–	–	+	+	+	+	+	+
6	–	–	–	–	+	–	+	+	+	+
7	–	–	–	–	+	+	+	+	+	+
10	–	–	–	–	+	+	+	+	+	+
11	–	–	–	–	+	+	+	+	+	+
12	–	–	–	–	+	+	+	+	+	+
13	–	–	–	–	–	+	+	+	+	+
14	–	–	–	+	+	+	+	+	+	+
15	–	–	–	–	–	–	+	+	+	+
18	–	–	–	–	+	–	+	+	+	+
Correct results (%)	100		11[d]		82[d]		100		96	

[a] Prepared by heating to boiling.
[b] – = negative test (zone ≤ 14.0 mm).
[c] + = positive test (zone > 14.0 mm).
[d] Significantly different from 100% at the $\alpha = 0.05$ level.

Table 3. Beta-lactam residues in heated milk detected by using PM Indicator Agar [a] and incubated at 55 ± 2°C

	Penicillin G (unit/mL)									
	0.000/mL sample		0.002/mL sample		0.004/mL sample		0.008/mL sample		0.012/mL sample	
Coll.	1	9	2	5	7	8	3	4	6	10
1	–[b]	–	–	+[c]	+	+	+	+	+	+
2	–	–	–	–	+	+	+	+	+	+
3	–	–	+	+	+	–	+	+	+	+
4	–	–	–	–	+	+	+	+	+	+
5	–	–	–	–	+	+	+	+	+	+
6	–	–	–	–	–	–	+	+	+	+
7	–	–	–	–	+	+	+	+	+	+
10	–	–	–	–	+	+	+	+	+	+
11	–	–	–	–	+	–	+	+	+	+
12	–	–	–	–	+	–	+	+	+	+
13	–	–	–	–	+	+	+	+	+	+
14	–	–	–	+	+	+	+	+	+	+
15	–	–	–	–	–	–	+	+	+	+
18	–	–	–	–	–	+	+	+	+	+
Correct results (%)	100		14[d]		71[d]		100		100	

[a] Prepared by autoclaving.
[b] – = negative test (zone ≤ 14.0 mm).
[c] + = positive test (zone > 14.0 mm).
[d] Significantly different from 100% at the $\alpha = 0.05$ level.

(**d**) *Broth medium D.*—Dissolve 17.0 g pancreatic digest of casein, 3.0 g papaic digest of soybean, 5.0 g NaCl, and 2.5 g K_2HPO_4 in H_2O and dil. to 1 L. Adjust if necessary so that after sterilization pH is 7.3 ± 0.2. (BBL Trypticase Soy Broth without Dextrose has been found satisfactory.)

16.C07 *Reagents and Apparatus*

(**a**) *Penicillin stock soln.*—See **16.132(a)**. (Difco PM Positive Controls, Penicillin G, and Penicillin Assays Inc. penicillin stds have been found satisfactory.)

(**b**) *Penicillinase (beta-lactamase).*—See **16.132(b)**. (Commercial Penase Discs (12.7 mm) have been found satisfactory.)

(**c**) *Filter paper disc, blank.*—Use S&S 740 E, 12.7 mm discs or discs of equiv. absorption performance qualities and purity.

(**d**) *Control discs.*—Prep. fresh daily from pos. control milks contg 0.008 IU penicillin/mL.

(**e**) *Petri dishes (plates).*—See **42.198(b)**.

16.C08 *Stock Culture of Test Organisms*

Maintain *B. stearothermophilus* ATCC 10149 on agar medium M (**c**), transferring to fresh slant weekly. Inoculate slant of agar medium M with test organism and incubate overnight at 55 ± 2° or 64 ± 2°. Inoculate three 300 mL erlenmeyers, each contg 150 mL broth medium D (**d**), with 1 loopful of test organism. Incubate at 55 ± 2° or 64 ± 2° and periodically make spore stains to det. extent of sporulation. When ca 80% sporulation has occurred (usually in 72 h), centrf. cell suspension 15 min at 5000 rpm. Decant supernate, resuspend cells in saline solution, **42.197(r)**, and recentrf. Repeat saline washing. Resuspend washed cells in 30 mL saline and store at 4°. Spore suspension will remain viable 6–8 months. Check viability periodically by prepn of trial test plates. (Com. prepd spore suspension has been found satisfactory.)

16.C09 *Preparation of Plates*

Inoculate aliquot of liquified agar medium B (**a**) or P (**b**), cooled to 55° or 64° with previously prepd spore suspensions. Adjust inoculum level to provide clear, readable zones of inhibition from penicillin-pos. control discs (**d**) after 3–4 h

Table 4. Comparison of assay media

Medium	50% Detection level (unit/mL)
Antibiotic Medium No. 4	0.00390[c]
PM Indicator Agar [a]	0.00351[c]
PM Indicator Agar [b]	0.00312[c]

[a] Prepared by heating to boiling.
[b] Prepared by autoclaving.
[c] Not significantly different at the $\alpha = 0.05$ level.

incubation at 55 ± 2° or 2–3 h incubation at 64 ± 2°. Pour 6 mL inoculated agar medium B or P into each plate, and let harden on flat, level surface. Use within 5 days.

16.C10 *Assay*

Screening.—With clean, dry forceps, touch paper disc to surface of well mixed milk and let milk be absorbed by capillary action. Drain excess milk by touching disc to inside surface of sample vessel. Immediately place disc on agar surface, pressing gently to ensure good contact. Identify each disc or section on which it is placed. Place control disc contg 0.008 IU penicillin/mL on plate. Invert plate and incubate at 55 ± 2° or 64 ± 2° until well defined zones of inhibition (17–20 mm) are obtained with the 0.008 IU/mL control. Examine plate for clear zone of inhibition surrounding discs. Clear zone of >14 mm indicates presence of inhibitory substances. Zones of ≤14 mm are read as neg. Confirm presence of inhibitor.

Confirming.—Heat test samples to 82° ≥2 min. Cool promptly to room temp. With clean, dry forceps, touch paper disc to surface of well mixed milk and let milk be absorbed by capillary action. Also fill penicillinase-impregnated disc or add 0.05 mL penicillinase to 5 mL sample and fill disc. Drain excess milk by touching disc to inside surface of sample vessel. Immediately place each disc on agar surface, pressing gently to ensure good contact. Place control disc contg 0.008 IU/mL on plate. Invert plate and incubate at 55 ± 2° or 64 ± 2° until well defined zones of inhibition (17–20 mm) are obtained with 0.008 IU/mL control. Examine plate for clear zone of inhibition (>14 mm) surrounding disc, indicating presence of inhibitory substance.

16.C11 *Interpretation*

Assay of test milk in screening and confirmatory test may produce following results:

(*1*) No zone around disc contg untreated milk in screening test is neg. test for inhibitory substances.

(2) Zone around disc contg untreated milk but no zone around disc contg penicillinase-treated milk in confirmatory test is pos. test for β-lactam residue.

(3) Clear zone of equal size around both discs in confirmatory test indicates presence of inhibitors other than β-lactam residues.

(*4*) Clear zone around penicillinase-treated milk substantially smaller than around untreated milk disc in confirmatory test indicates presence of β-lactam residues as well as another inhibitor(s).

Penicillin-pos. control soln at 0.008 IU/mL

Table 5. Screening tests for beta-lactam residues in unheated milk incubated at 55 ± 2°C

	Penicillin G (unit/mL)									
	0.000/mL sample		0.002/mL sample		0.004/mL sample		0.008/mL sample		0.012/mL sample	
Coll.	1	9	2	5	7	8	3	4	6	10
1	–[a]	–	–	–	+[b]	+	+	+	+	+
2	–	–	+	–	+	+	+	+	+	+
3	–	–	–	–	+	+	+	+	+	+
4	–	–	–	–	–	–	+	+	+	+
5	–	–	+	+	+	+	+	+	+	+
6	–	–	+	+	+	+	+	+	+	+
7	–	–	–	–	–	–	+	+	+	+
8	–	–	–	–	+	+	+	+	+	+
9	–	–	–	–	+	+	+	+	+	+
10	–	–	–	–	+	+	+	+	+	+
11	–	–	–	–	+	+	+	+	+	+
12	–	–	–	–	+	+	+	+	+	+
13	–	–	–	–	–	+	+	+	+	+
14	–	–	–	–	+	+	+	+	+	+
15	–	–	+	+	+	+	+	+	+	+
16	–	–	+	+	+	+	+	+	+	+
Correct results (%)	100		28[c]		84[c]		100		100	

[a] – = negative test (zone ≤ 14.0 mm).
[b] + = positive test (zone > 14.0 mm).
[c] Significantly different from 100% at the α = 0.05 level.

Table 6. Screening tests for beta-lactam residues in unheated milk incubated at 64 ± 2°C

	Penicillin G (unit/mL)									
	0.000/mL sample		0.002/mL sample		0.004/mL sample		0.008/mL sample		0.012/mL sample	
Coll.	1	9	2	5	7	8	3	4	6	10
1	−[a]	−	−	−	+[b]	−	+	+	+	+
2	−	−	−	−	−	+	+	+	+	+
3	−	−	−	−	+	+	+	+	+	+
4	−	−	−	−	+	+	+	+	+	+
5	−	−	+	+	+	+	+	+	+	+
6	−	−	−	−	+	+	+	+	+	+
7	−	−	−	−	−	+	+	+	+	+
8	−	−	−	−	−	−	+	+	+	+
9	−	−	−	−	−	−	+	+	+	+
10	−	−	−	−	−	−	+	+	+	+
11	−	−	−	−	+	+	+	+	+	+
12	−	−	−	−	+	−	+	+	+	+
13	−	−	−	−	−	−	+	+	+	+
14	−	−	−	−	−	−	+	+	+	+
15	−	−	−	−	+	−	+	+	+	+
16	−	−	−	−	+	−	+	+	+	+
Correct results (%)	100		6[c]		50[c]		100		100	

[a] − = negative test (zone ≤ 14.0 mm).
[b] + = positive test (zone > 14.0 mm).
[c] Significantly different from 100% at the $\alpha = 0.05$ level.

should produce clear, well defined zones of inhibition (17–20 mm). If no zone of inhibition is produced by penicillin-pos. control, test sensitivity is not adequate and test should be repeated.

Results and Discussion

Fourteen collaborators participated in part A and 16 in part B of the study. Tables 1–3 present the collaborative data from part A, in which the equivalency of the 2 agar media was tested. The percentage of correct results is given for each pair of samples. The lowest concentration not significantly different from 100% at the $\alpha = 0.05$ level was 0.008 unit/mL. For purposes of comparison, the ED50 was used. As demonstrated in Table 4, the detectable concentration of the beta-lactam inhibitor (penicillin G) did not differ significantly at the $\alpha = 0.05$ level for the 2 agar media; therefore, either medium is suitable for use in the assay. In addition, no false positives were reported when either medium was used.

Because of the equivalency of the assay media, only one (PM Indicator Agar) was used in the collaborative study. Tables 5–8 present the data obtained by each collaborator in the screening and confirmatory tests at 55 and 64°C. The percentage of correct results is given for each pair of samples. The lowest concentration not significantly different from 100% at the $\alpha = 0.05$ level was 0.008 unit/mL. No false positives were reported at either incubation temperature. All samples found positive by the confirmatory test were correctly identified with a commercial Penase disc as having a beta-lactam inhibitor. Table 9 compares the concentration of beta-lactam inhibitor detectable by the screening and confirmatory tests at 55 and 64°C. Although statistical analysis demonstrated significant differences between screening and confirmatory tests and assays, these small statistical differences were not considered to be of practical significance because the test was designed to be qualitative rather than quantitative.

The 50% concentration of beta-lactam (penicillin G) calculated from the data presented in the collaborative study (7) on the current official method (3) was 0.005 unit/mL. The 50% concentrations demonstrated in this collaborative study ranged from 0.003 to 0.005 unit/mL. Thus the methods are of equal sensitivity.

Recommendation

These collaborative studies demonstrated that the *Bacillus stearothermophilus* disc assay method can be used to detect and identify a beta-lactam

Table 7. Confirmatory tests for beta-lactam residues in heated milk incubated at 55 ± 2°C

	Penicillin G (units/mL)									
	0.000/mL sample		0.002/mL sample		0.004/mL sample		0.008/mL sample		0.012/mL sample	
Coll.	1	9	2	5	7	8	3	4	6	10
1	–[a]	–	–	–	+[b]	–	+	+	+	+
2	ND[c]	ND	–	–	+	+	+	+	+	+
3	ND	ND	–	–	+	+	+	+	+	+
4	–	–	–	–	–	–	+	+	+	+
5	–	–	+	–	+	+	+	+	+	+
6	–	–	–	–	–	–	+	+	+	+
7	ND	ND	ND	ND	–	+	+	+	+	+
8	–	–	–	–	–	+	+	+	+	+
9	–	–	–	–	+	+	+	+	+	+
10	–	–	–	–	–	–	+	+	+	+
11	–	–	–	–	+	+	+	+	+	+
12	–	–	–	–	+	+	+	+	+	+
13	ND	ND	ND	ND	–	–	+	+	+	+
14	ND	ND	ND	ND	–	–	+	+	+	+
15	–	–	–	–	+	+	+	+	+	+
16	ND	ND	–	+	+	+	+	+	+	+
Correct results (%)	100		8[d]		59[d]		100		100	

[a] – = negative test (zone ≤ 14.0 mm).
[b] + = positive test (zone > 14.0 mm).
[c] ND = not done.
[d] Significantly different from 100% at the $\alpha = 0.05$ level.

Table 8. Confirmatory tests for beta-lactam residues in heated milk incubated at 64 ± 2°C

	Penicillin G (units/mL)									
	0.000/mL sample		0.002/mL sample		0.004/mL sample		0.008/mL sample		0.012/mL sample	
Coll.	1	9	2	5	7	8	3	4	6	10
1	–[a]	–	–	–	–	–	+[b]	+	+	+
2	ND[c]	ND	ND	ND	–	–	+	+	+	+
3	ND	ND	ND	ND	+	+	+	+	+	+
4	–	–	–	–	+	–	+	+	+	+
5	–	–	–	–	–	–	+	+	+	+
6	–	–	–	–	–	–	+	+	+	+
7	ND	ND	ND	ND	–	–	+	+	+	+
8	–	–	–	–	–	–	+	+	+	+
9	–	–	–	–	–	–	+	+	+	+
10	–	–	–	–	–	–	+	+	+	+
11	–	–	–	–	+	+	+	+	+	+
12	–	–	–	–	+	+	+	+	+	+
13	ND	ND	ND	ND	ND	ND	+	+	+	+
14	ND	ND	ND	ND	ND	ND	+	+	+	+
15	–	–	–	–	–	–	+	+	+	+
16	ND	ND	ND	ND	–	ND	+	+	+	+
Correct results (%)	100		0[d]		26[d]		100		100	

[a] – = negative test (zone ≤ 14.0 mm).
[b] + = positive test (zone > 14.0 mm).
[c] ND = not done.
[d] Significantly different from 100% at the $\alpha = 0.05$ level.

Table 9. 50% Detection level

	Screening test		Confirmatory test	
Penicillin G	55°C	64°C	55°C	64°C
Unit/mL	0.0026[a]	0.0038[b]	0.0035[b]	0.0053[c]

[a] Significantly lower at the $\alpha = 0.05$ level.
[b] Not significantly different at the $\alpha = 0.05$ level.
[c] Significantly higher at the $\alpha = 0.05$ level.

inhibitor rapidly, precisely, and accurately. It is recommended that this method be adopted official first action.

Acknowledgments

The Associate Referee gratefully acknowledges the assistance of the following collaborators: A. Appelt and W. Kelley, South Dakota State Chemical Laboratory, Vermillion, SD; E. Boone, P. Dombrowski, C. Payton, and D. Seiz, Illinois Department of Public Health, Springfield, IL; H. Bryant, L. Burroughs, and D. Herbst, National Center for Antibiotics Analysis, Food and Drug Administration, Washington, DC; K. Coleman, Dairymen, Inc., Spartanburg, SC; M. Cupery, Kraft, Inc., Beaver Dam, WI; R. Cyr and R. Mullen, Vermont Department of Agriculture, Montpelier, VT; P. Donahue and R. Ginn, Dairy Quality Control Institute, Minneapolis, MN; S. Farmer and J. Heady, Missouri Division of Health, Jefferson City, MO; H. Grant, Jr, Florida Department of Agriculture, Winter Haven, FL; J. Hawkins, Illinois Department of Public Health, Carbondale, IL; M. Misup and N. Ondrus, Illinois Department of Public Health, Chicago, IL; L. Morris, Difco Laboratories, Detroit, MI; R. Share and M. Stern, Kraft, Inc., Chicago, IL; R. Williams, Oregon Department of Agriculture, Salem, OR.

References

(1) *Grade "A" Pasteurized Milk Ordinance. 1978 Recommendations of the U.S. Public Health Service*, Food and Drug Administration, Washington DC, p. 25
(2) *Standard Methods for the Examination of Dairy Products* (1978) 14th Ed., American Public Health Association, Washington, DC, pp. 141–150
(3) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **16.131–16.136**
(4) Duncan, D. B. (1955) *Biometrics* **11,** 1–42
(5) Finney, D. J. (1978) *Statistical Methods in Biological Assay*, 3rd Ed., Macmillan Publishing Co., Inc., New York, NY
(6) Pearson, E. S., & Hartley, H. O. (1970) *Biometrika Tables for Statisticians*, Vol. 1, 3rd. Ed., Cambridge University Press, Cambridge, England, pp. 83–84, 228
(7) Ouderkirk, L. A. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 985–988

VITAMINS AND OTHER NUTRIENTS

Determination of Tocopherols (Vitamin E) by Reduction of Cupric Ion

EMILIO S. CONTRERAS-GUZMÁN and FREDERICK C. STRONG III
Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos e Agrícola, Caixa Postal 6121, 13100 Campinas, São Paulo, Brasil

This research details the principal characteristics of a new chemical reaction for the determination of tocopherols (vitamin E). The proposed technique is based on the reduction of Cu(II) to Cu(I) by the tocopherols. The reduced ion is then available to form a complex with 2,2′-biquinoline (cuproine) and with 2,9-dimethyl - 4,7-diphenyl-1,10-phenanthroline (bathocuproine), which have maximum absorbances at 545 and 478 nm, respectively. One mole of tocopherol reduces 2 moles of copper. When urea is used as a catalyst in the reaction, α-tocopherol and γ-tocopherol react completely in 10 s; δ-tocopherol reacts in 90 s. The reaction is not affected by exposure to light. The method was compared with 2 other methods: reduction of Fe(III) to Fe(II) and complexation with 2,2′-bipyridine (conventional Emmerie-Engel method), and reduction of the free radical 1,1′-diphenyl-2-picrylhydrazyl (DPPH) to the corresponding hydrazine. Absorptivities of the complexes $Cu(cuproine)_2^+$, $Cu(bathocuproine)_2^+$, $Fe(bipyridine)^{++}$, and DPPH, in terms of concentration of tocopherol were: 30.89, 61.87, 39.91, and 48.86 L g^{-1} cm^{-1}, respectively.

The early work of Emmerie and Engel (1, 2) has become a standard procedure for the determination of tocopherol (vitamin E), because it is simple and rapid. In the original procedure, ferric ion is reduced to ferrous ion which then complexes with 2,2′-bipyridine. The product has a strong absorbance at 520 nm. Ferrous ion can also be complexed with 4,7-diphenyl-1,10-phenanthroline or 2,4,6-tripyridyl-5-tetrazine, according to the modification by Tsen (3).

The reaction with ferric ions is affected by interferences due to traces of reductants or oxidants, time of reaction, and especially exposure to light. The modification by Tsen improved the sensitivity but did not overcome the susceptibility of ferric ion to photoreduction.

The lower sensitivity with the original complex (2,2′-bipyridine) was not a major problem because vitamin E exists in relatively large quantities in natural products. Many researchers continue to use the original reaction; however, they stabilize it with phosphoric acid as recommended by Tsen.

Another method was proposed by Glavind and Holmer (4), following the suggestions of Blois (5). They recommended the use of the radical 1,1′-diphenyl-2-picrylhydrazyl (DPPH) to oxidize tocopherols. According to Pryor (6), the free radical DPPH is reduced to the corresponding hydrazine when it reacts with hydrogen donors of the type found in tocopherols, phenols, and synthetic antioxidants. Contreras (7) described a method for determining ethoxyquin in fish meal and showed that the principal interference was α-tocopherol in the tissues. Boguth and Repges (8) also developed a method for tocopherols with DPPH.

A variety of chemical compounds have been used for detecting tocopherols by paper chromatography and thin layer chromatography (TLC). In his 2 reviews, Bunnell (9, 10) mentions the use of 2,6-dichloroquinone chlorimide, phosphomolybdic acid, iodine, silver nitrate, antimony pentachloride, and other compounds. The same author reports that ceric sulfate was used to determine purity of pharmaceutical preparations.

Semenova and Kutnetsow (11) tested *p*-phenylenediamine, chromotropic acid, and titanium sulfate in sulfuric acid, and found that these reagents permitted distinguishing the different tocopherols colorimetrically.

The present work is based on the assumption that, if the tocopherols are so active in reducing ferric ions, they would also reduce other ions which might not have the disadvantages of ferric ions. Such substances should have the following characteristics: They should be rapidly reduced by tocopherols, should be reasonably soluble in organic solvents, and should permit the determination of the reduced ion simply and sensitively. The cupric ion, as its nitrate or acetate, was considered as possibly fulfilling all of these requirements.

In the literature consulted, we found no re-

This work is a portion of a thesis to be submitted by E. S. Contreras-Guzmán in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Science at the Universidade Estadual de Campinas.

Received November 6, 1981. Accepted February 22, 1982.

0004-5756/82/6505-1215-07$01.00

corded use of cupric ion in the qualitative or quantitative evaluation of tocopherols. Preliminary experiments indicated that acids inhibited and bases accelerated the reduction of cupric ions by tocopherols; however, bases caused precipitation. Urea did not show this inconvenience and therefore we investigated its use as a catalyst.

The reaction between tocopherols and cupric ions was studied and compared with the original method of Emmerie and Engel (1, 2) and with the reduction of the free radical DPPH (5).

Experimental

Materials

(a) *Complexing agents.*—2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline (bathocuproine); 2,2′-biquinoline (cuproine); 2,2′-bipyridine; 1,1′-diphenyl-2-picrylhydrazyl (DPPH).

(b) *Cupric and ferric reagents.*—Cupric nitrate, $Cu(NO_3)_2 \cdot 3\ H_2O$; cupric acetate, $(CH_3COO)_2Cu \cdot H_2O$; ferric chloride, $FeCl_3 \cdot 6\ H_2O$.

(c) *Tocopherols.*—all-rac-α-Tocopherol for biochemical use, 99%, Merck; D-γ-tocopherol and D-δ-tocopherol isolated from natural sources (11).

(d) *Toluene.*—To purify, add 50 mL saturated cupric acetate in ethanol to 1 L toluene and let solution stand overnight. Wash solution twice with distilled water and dry with anhydrous Na_2SO_4. Distill and collect middle fraction of 750 mL.

(e) *Ethanol.*—To purify, add 5 g KOH to 1 L absolute ethanol and let solution stand overnight. Distill and collect middle fraction of 750 mL. To distillate, add 0.1 g cupric acetate and reflux mixture 1 h. Then distill again.

(f) *0.50% Cuproine in toluene.*—Store in amber bottle in freezer.

(g) *0.25% Bathocuproine in toluene.*—Store in amber bottle in freezer.

(h) *2.5% Urea in absolute ethanol.*

(i) *0.1% Cupric nitrate in absolute ethanol.*

(j) *DPPH solutions.—Stock solution:* 0.04% DPPH in absolute ethanol. Store in amber bottle in freezer. *Working solution:* Dilute stock solution to prepare 0.004% DPPH in absolute ethanol. Discard after analysis.

(k) *0.5% Ferric chloride in absolute ethanol* (12).

(l) *0.5% 2,2′-Bipyridine in absolute ethanol* (12). —Store in amber bottle in freezer.

(m) *Tocopherol solutions.—Stock solution:* 0.1% α-Tocopherol, D-γ-tocopherol, and D-δ-tocopherol in absolute ethanol. Store in amber bottle in freezer. *Working solution:* Dilute 0.1% solutions to prepare 0.005% solutions of tocopherols. Discard after analysis.

(n) *Complexing reagent.*—0.50% Cuproine in toluene–2.5% urea in absolute ethanol–0.1% cupric nitrate in absolute ethanol (0.5 + 2.5 + 1) or 0.25% bathocuproine in toluene–2.5% urea in absolute ethanol–0.1% cupric nitrate in absolute ethanol (0.5 + 2.5 + 1). Prepare fresh for each determination.

(o) *Copper standard solutions.—Stock solution:* 0.125% (1.25 mg Cu/mL). Prepare by dissolving electrolytic copper in HNO_3. Evaporate solution to eliminate excess acid, dissolve residue in water, and dilute to prepare solution containing 1.25 mg Cu/mL. *Working solution:* 25 μg Cu/mL. Dilute 10 mL stock solution to 500 mL with absolute ethanol. Discard working solution after analysis.

Procedure

Reaction velocity experiments using cupric ion.—Prepare mixture of 1 mL tocopherol working solution and 3 mL complexing reagent. Quickly place solution in Zeiss M4 QIII spectrophotometer and read absorbance at 10 s intervals. For uncatalyzed reaction, prepare complexing reagent by using 2.5 volumes of pure ethanol instead of 2.5% urea solution.

Reaction between cupric ions and excess of α-tocopherol.—Dilute 0.1, 0.2, 0.4, 0.6, and 0.8 mL portions of copper working solution to 1 mL with ethanol. To each dilution, add 0.5 mL solution (f) or (g), 1.5 mL solution (h), and 1 mL α-tocopherol stock solution. Shake tubes and read absorbance after 10 min at 545 nm (cuproine) or at 478 nm (bathocuproine).

Reaction between tocopherols and excess of cupric ions.—Add 0.1, 0.2, 0.4, 0.8, and 1 mL portions of each of the 3 tocopherol working solutions to 3 mL complexing reagent. Adjust volume of each solution to 4 mL with ethanol. Measure absorbance within 2–15 min.

Reaction between tocopherols and free radical DPPH.—Add 0.1, 0.2, 0.4, 0.8, and 1 mL portions of each of the 3 tocopherol working solutions to 3 mL DPPH working solution. Adjust volume of each solution to 4 mL with ethanol. For reaction velocity experiments, take readings every 10 s; take other measurements after 15 min. Prepare reagent blank by substituting 1 mL pure ethanol for tocopherol working solution. Difference between readings for blank and sample corresponds to DPPH consumed by tocopherol.

Reaction between tocopherols and ferric ions (Em-

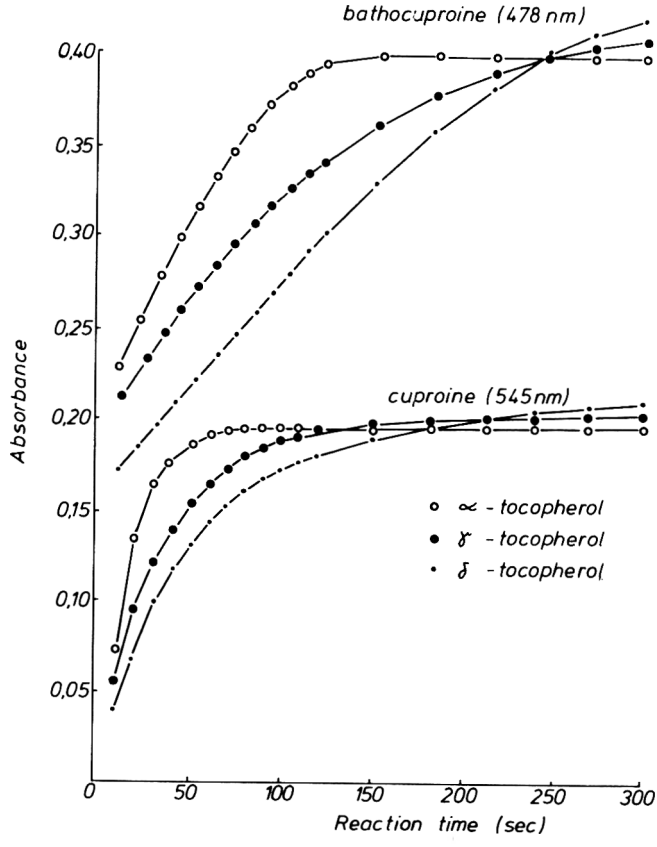


Figure 1. Reaction rate of tocopherols with cupric ion in presence of cuproine and bathocuproine.

merie-Engel method).—Use conditions recommended by Müller Mulot (12), adjusting final volume to 4 mL.

Results and Discussion

Reaction Rate Studies

Figures 1 and 2 demonstrate the variation of absorbance with time for the reaction with copper; Figures 3 and 4 show the variation for reaction with the ferric ion and DPPH, respectively. In all of the reactions, velocity was a function of the number of methyl groups in the chroman ring, and was a maximum for the 5,7,8-trimethyl tocol (α-tocopherol) and a minimum for the 8-methyl tocol (δ-tocopherol). The reaction with DPPH was the slowest of all, requiring more than 10 min for δ-tocopherol to react completely.

The catalytic effect of urea is clearly evident when Figures 1 and 2 are compared. In the presence of urea, using cuproine, α-tocopherol and γ-tocopherol were evaluated in less than 10 s; δ-tocopherol could be evaluated in 90 s.

The curves for the complex with bathocuproine also showed a notable increase in reaction velocity, allowing the δ-tocopherol to be determined after 120 s.

During this research, it was not possible to establish whether the urea acts as a proton acceptor, or forms a transitory complex with cupric ions that favors their reduction by tocopherols, or if its function is of some other nature. In any case, urea is shown to be a very efficient catalyst and was incorporated in all subsequent experiments. A test showed that urea is without effect, however, in the reaction of ferric ions with tocopherols.

The fact that all tocopherols can be determined after 2 min by the catalyzed reaction presented here would be of great value in the analysis of vegetable oils. It is doubtful whether the analysis of oils by the Emmerie-Engel procedure results in complete reaction of δ-tocopherol in the 2.5 min conventionally used. Müller Mulot (12) and others have recommended that the time of reaction be increased to 10 min.

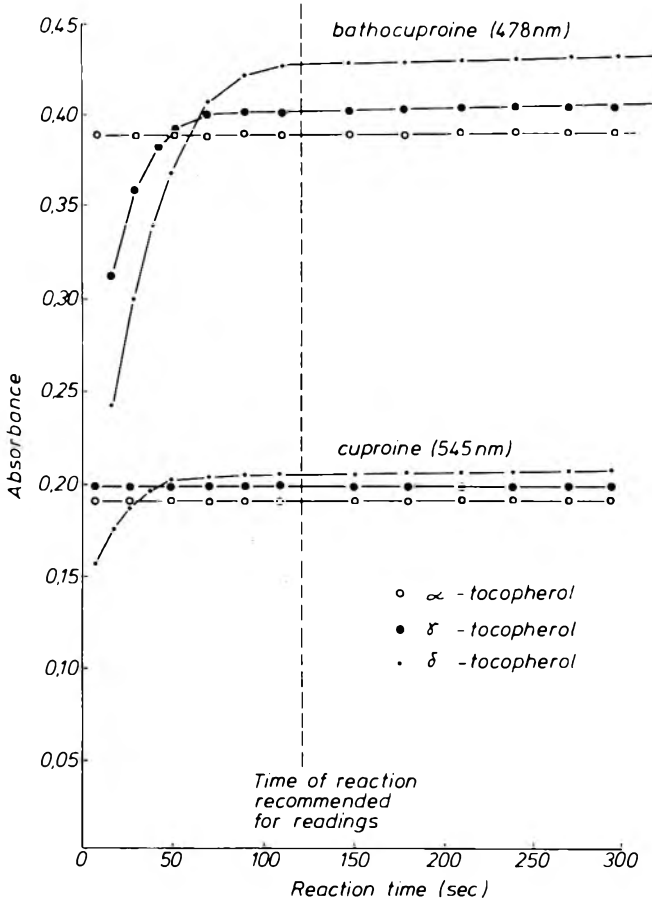


Figure 2. Reaction rate of tocopherols with cupric ion in presence of cuproine and bathocuproine, catalyzed by urea.

Experiments at Equilibrium Conditions

Absorbance data for various cupric ion concentrations reacting with excess α-tocopherol and cuproine or bathocuproine are shown in Table 1. Calculating molar absorptivities on the basis of copper gives 6590 (cuproine) and 13 800 (bathocuproine), which are in accordance with values cited by Schilt (13), 6450 and 14 200, respectively. It should be noted that the data collected by Schilt were obtained by reducing the cupric ion with hydroxylamine in buffered solution at pH 5–7 and extracting the cuprous complex with isoamyl or hexyl alcohol. This agreement of absorptivities demonstrates that cupric ions can be reduced in an ethanolic medium by a hydrophobic reductant like α-tocopherol. The similarity in the molar absorptivity data also shows quantitative reduction.

Table 1. Variation of absorbance [a] with copper concentration in presence of excess α-tocopherol and complexing reagents

Concn, g Cu^{++}/mL	Cuproine, *A* at 545 nm	Bathocuproine, *A* at 478 nm
0.625	0.065	0.133
	0.066	0.138
1.25	0.129	0.273
	0.133	0.274
2.50	0.257	0.541
	0.259	0.542
5.00	0.518	1.085
	0.522	1.090
10.00	1.035	—
	1.040	—
a_{Cu}	103.7	217.4
ϵ_{Cu}	6590	13 810

[a] Duplicate analyses were carried out with each complexing reagent at each concentration.

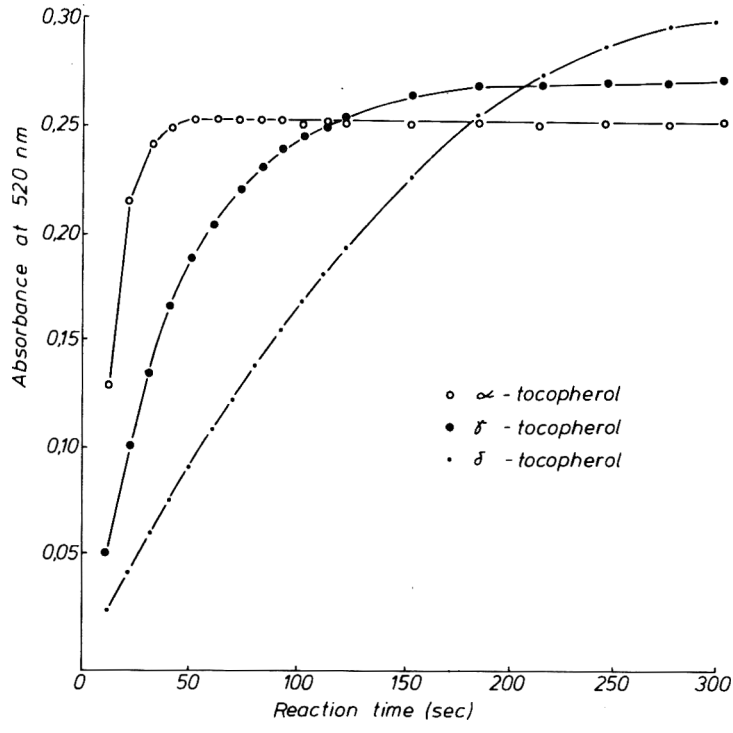


Figure 3. Reaction rate of tocopherols with ferric ion in presence of 2,2′-bypyridine (Emmerie-Engel reagent).

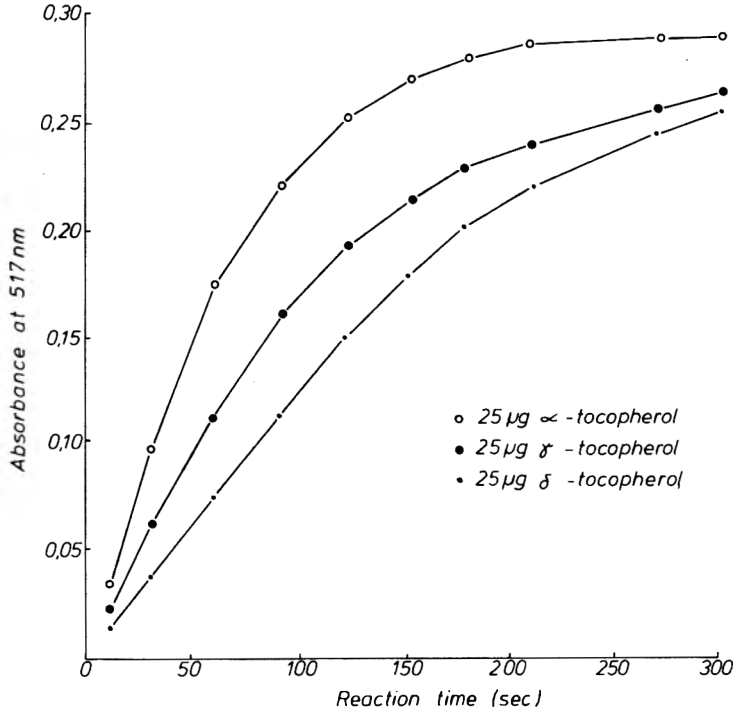


Figure 4. Reaction rate of tocopherols with 1,1′-diphenyl-2-picrylhydrazyl free radical.

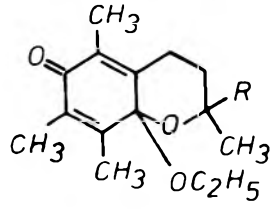
Table 2. Spectrophotometric data for four reactions for determination of α-tocopherol

Concn, μg α-T/mL	$Cu(cuproine)_2^+$, A at 545 nm	$Cu(bathocuproine)_2^+$, A at 478 nm	$Fe(bipyridine)^{++}$, A at 520 nm	DPPH,[a] ΔA at 517 nm
1.25	0.044	0.089	0.046	0.068
	0.047	0.089	0.052	0.068
2.50	0.076	0.164	0.090	0.142
	0.080	0.170	0.108	0.142
5.00	0.156	0.312	0.190	0.238
	0.160	0.319	0.212	0.248
10.00	0.308	0.621	0.388	0.508
	0.310	0.634	0.405	0.512
12.50	0.386	0.791	0.492	0.650
	0.388	0.791	0.508	0.652
b_0[b]	0.00342	0.01018	−0.00050	0.01061
b_1[b]	0.03089	0.06187	0.03991	0.04886
r	0.9998	0.9999	0.9991	0.9993
s_e	0.0034	0.0047	0.0097	0.0101
a	30.89	61.87	39.91	48.86
ϵ	13300	26650	17190	21040

[a] See "*Procedure*" for explanation of data for DPPH.
[b] For the regression equation $y = b_0 + b_1 x$.

In Table 2, columns 1 and 2 give the results of the complementary experiment in which various measured quantities of α-tocopherol were reacted with an excess of cupric ions (this corresponds to the analytical application of the reaction). In the other columns, the corresponding data are tabulated for the reactions with ferric ions–2,2′-bipyridine and with DPPH.

When the molar absorptivities for cuproine and bathocuproine, calculated on the basis of α-tocopherol (MW 430.72), were compared with those in Table 1, based on copper, we found a molar reaction ratio for Cu^{++}/α-T of 2/1: 13300/6590 = 2.02 and 25650/13800 = 1.93. Goodhue and Risley (14) showed that the product of the oxidation of α-tocopherol by benzoyl peroxide in alcoholic solutions is the hemiacetal of the α-tocopheroxide. Because formation of this product requires 2 electrons, which is also shown above for our reaction, and the solvent used in our procedure was absolute ethanol (plus a small amount of toluene), we propose that the product is the same.



The comparison of the reactions using cupric ions with the 2 optional reactions in the literature, as presented in Table 2, reveals that the sensitivities are $Cu(bathocuproine)_2^+$ > DPPH > $Fe(bipyridine)^{++}$ > $Cu(cuproine)_2^+$. Although $Cu(cuproine)_2^+$ is least in sensitivity, its more favorable coefficient of correlation and standard error of estimate make it suitable for use in the determination of total tocols in vegetable oils, which usually have relatively large amounts of tocopherols. For lower concentrations, the $Cu(bathocuproine)_2^+$ complex is recommended. In these experiments, the reactions were carried out under normal laboratory illumination, a combination of daylight and artificial light, and all glassware was colorless. Absorbances of solutions of the copper complex remained constant for hours.

The application of the new reaction to the study of tocopherols and tocotrienols in fresh and mature corn has been reported in a preliminary form (XIV Congreso Latinoamericano de Química, San José, Costa Rica, February 1981). A more extensive publication on analysis of cereals and vegetable oils is in preparation.

REFERENCES

(1) Emmerie, A., & Engel, C. (1938) *Nature* **142**, 873
(2) Emmerie, A., & Engel, C. (1938) *Rec. Trav. Chim.* **57**, 1351–1355
(3) Tsen, C. C. (1961) *Anal. Chem.* **33**, 849
(4) Glavind, J., & Holmer, G. (1967) *J. Am. Oil. Chem. Soc.* **44**, 539
(5) Blois, M. S. (1958) *Nature* **181**, 1199
(6) Pryor, W. A. (1966) *Free Radicals*, 1st Ed., McGraw Hill Book Co., New York, NY, pp. 26–27, 326–327
(7) Contreras-Guzmán, E. S. (1970) *Bol. Cient. Inst. Fom.*

Pesquero, No. 12, pp. 1–39.
(8) Boguth, W., & Repges, R. (1969) *Int. Z. Vitaminforsch.* **39,** 289
(9) Bunnell, H. R. (1967) in *The Vitamins*, P. G. György & W. N. Pearson (Eds), Academic Press, New York, NY, Vol. VI, pp. 261–314
(10) Bunnell, H. R. (1971) *Lipids* **6,** 245
(11) Semenova, L. I., & Kutnetsov, D. I. (1975) *Prikl. Biokhim. Mikrobiol.* **11,** 922–926; *Chem. Abstr.* (1976) **84,** 56022k
(12) Müller Mulot, W. (1976) *J. Am. Oil Chem. Soc.* **53,** 732
(13) Schilt, A. A. (1969) *Analytical Applications of 1,10-Phenanthroline and Related Compounds*, 1st Ed., Pergamon Press, Inc., London, UK, pp. 3–9, 69–73
(14) Goodhue, C. T., & Risley, H. A. (1965) *Biochemistry* **4,** 854–858

Enzymatic Determination of Cholesterol in Egg Yolk

CHIH-SHANG J. SHEN, ISABEL S. CHEN, and ALAN J. SHEPPARD
Food and Drug Administration, Division of Nutrition, Washington, DC 20204

The quantitative determination of cholesterol in egg yolk by using an enzymatic test kit is described. Cholesterol in the egg yolk is extracted with other lipid components by methylene chloride-methanol (2 + 1) and is enzymatically determined after saponification of the lipid extract. The method is relatively rapid, simple, and accurate and gives results which agree with those obtained by using a gas-liquid chromatographic (GLC) method. The mean cholesterol content of egg yolk determined by the enzymatic and GLC methods was 1237 and 1240 mg/100 g, respectively.

The increased need for a rapid, simple method for determining cholesterol in foods has become apparent in government regulatory and food industry quality assurance laboratories with the advent of voluntary food labeling regulations (1). The cholesterol labeling requirement was prompted primarily by the increasing concern about the health implications of dietary cholesterol (2–7).

A variety of methods, including chemical (8, 9), gas-liquid chromatographic (GLC) (10–14), and high performance liquid chromatographic (HPLC) (15), are presently available for the determination of cholesterol in foods. Derivatization of cholesterol is generally required for GLC and HPLC. The choice of method usually depends on the type of sample analyzed, the accuracy and sensitivity required, and the availability of instrumentation. For egg and egg products, the official AOAC method is a titrimetric method using sodium thiosulfate (8). However, this procedure is somewhat slow and tedious, and lacks specificity. GLC, because of its specificity, sensitivity, and accuracy, has been the preferred method in recent years (9, 10).

Several rapid, simple enzymatic methods using cholesterol oxidase have been developed for the determination of serum cholesterol (16–21). These methods differ mainly in the coupled indicator reaction used to measure the hydrogen peroxide generated from the cholesterol oxidase reaction. These methods have not been applied to the determination of cholesterol in foods to any appreciable extent. An enzymatic method (18), using a test kit, has been employed satisfactorily for the determination of cholesterol in milk (22) and tissue (23). In the present study, this method was evaluated to determine its suitability for quantitative determination of cholesterol in egg yolk. Cholesterol was extracted along with other lipid components by methylene chloride–methanol (2 + 1) (24). After saponification of the lipid extract, cholesterol was measured by both the enzymatic method and a GLC method (12), and the results were compared.

METHODS

Apparatus and Reagents

(a) *Spectrophotometer.*—Perkin-Elmer Model 402 ultraviolet-visible.

(b) *Enzymatic test kit.*—Obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN. Contains 3 bottles of solution: solution 1 consists of ammonium phosphate buffer (0.8 mol/L, pH 7.0), methanol (2.6 mol/L), and catalase (2.3×10^6 U/L); solution 2 consists of acetylacetone (0.05 mol/L), methanol (0.3 mol/L), and stabilizers; and solution 3 is a cholesterol oxidase suspension (12×10^3 U/L).

(c) *Reagent mixture.*—Thoroughly mix 3 parts solution 1 and 2 parts solution 2. Store in brown bottle.

(d) *Cholesterol standard solutions.—Stock solution.*—Accurately weigh 100 mg pure cholesterol (Boehringer-Mannheim Biochemicals) and quantitatively transfer to 100 mL volumetric flask with isopropanol; dilute to volume with isopropanol. Prepare series of cholesterol standard solutions of the following concentrations: 100, 200, 400, 500, 600, 800, and 1000 μg/mL isopropanol.

(e) *Trifluoride–methanol solution.*—14% (w/v) (Sulpelco Inc., Bellefonte, PA).

(f) *Butyric anhydride and pyridine.*—Eastman Kodak Co., Rochester, NY.

Sample Preparation

Carefully separate egg yolk from egg white. Take representative sample of egg yolk containing ca 1 g fat and transfer to homogenization vessel. Extract total lipid from sample, using

This paper was presented in part at the 65th Annual Meeting of the Federation of American Societies for Experimental Biology, April 12–17, 1981, at Atlanta, GA.
Received January 8, 1982. Accepted February 8, 1982.

0004-5756/82/6505-1222–03$01.00

Table 1. Cholesterol content (mg/100 g) of egg yolk as measured by enzymatic and GLC methods

Sample No.	Enzymatic[a]	GLC[b]
1	1129.8 ± 15.3 (1.4)	1133.4 ± 75.2 (6.7)
2	1143.5 ± 11.1 (1.0)	1159.0 ± 37.8 (3.3)
3	1305.0 ± 13.2 (1.0)	1282.6 ± 36.5 (2.8)
4	1330.2 ± 44.9 (3.4)	1306.1 ± 10.8 (0.8)
5	1068.8 ± 24.9 (2.3)	1049.5 ± 14.0 (1.3)
6	1304.4 ± 15.7 (1.2)	1272.7 ± 37.0 (2.9)
7	1091.0 ± 24.9 (2.3)	1091.8 ± 42.6 (3.9)
8	1271.2 ± 17.4 (1.4)	1293.2 ± 20.2 (1.6)
9	1246.6 ± 20.5 (1.6)	1213.7 ± 24.7 (2.0)
10	1349.8 ± 20.7 (1.5)	1373.3 ± 33.6 (2.4)
11	1324.0 ± 27.9 (2.1)	1379.5 ± 58.9 (4.3)
12	1282.0 ± 34.4 (2.7)	1325.4 ± 29.2 (2.2)
$\bar{X}$	1237.2	1240.0
SD	100.6	109.3

[a] Each value is the mean of 8 determinations ± SD (CV, %).
[b] Each value is the mean of 4 determinations ± SD (CV, %).

methylene chloride–methanol (2 + 1) (24). Saponify lipid extract and prepare fatty acid methyl esters (25). The hexane solution obtained (25) contains free cholesterol and fatty acid methyl esters. Take aliquot of hexane solution, dry under stream of nitrogen, and then dilute to original volume with isopropanol. This isopropanol solution, which contains free cholesterol, is used as the sample solution in the assay procedure.

Enzymatic Assay

Pipet 5 mL reagent mixture and 0.4 mL sample solution or cholesterol standard solution into test tube (20 × 150 mm). Thoroughly mix contents of test tube. Pipet 2.5 mL solution into each of 2 screw-cap test tubes (13 × 100 mm). To first test tube (blank), add 0.02 mL water and mix well. To second test tube (sample), add 0.02 mL cholesterol oxidase suspension and mix thoroughly. Cap test tubes and incubate 90 min in 37°C water bath. Remove test tubes from water bath and let cool to room temperature (ca 1 h). Transfer contents of test tubes to matched 1 cm quartz cells and place in spectrophotometer. Read absorbance of sample against blank at 405 nm to obtain absorbance (A). Determine cholesterol concentration of sample solution from standard curve, which is constructed by graphically plotting A vs μg/mL for each level of cholesterol standard solution. Linear range of standard curve obtained is similar to that reported by other workers (18, 22, 23).

Calculation

Cholesterol content, mg/100 g product = $C \times DF \times 100/S$, where C = μg cholesterol/mL sample solution (obtained from standard curve), DF = dilution factor, and S = sample weight, g.

Gas-Liquid Chromatography

Prepare butyrate derivative of cholesterol, using portion of hexane solution containing free cholesterol, and determine cholesterol content by GLC method described by Sheppard et al. (12).

Results and Discussion

The enzymatic method used in the present study consists of 2 enzymatic reactions, catalyzed by cholesterol oxidase and catalase, and the Hantzsch reaction (18). Cholesterol is oxidized by cholesterol oxidase to cholesterone. The hydrogen peroxide produced oxidizes methanol to formaldehyde in the reaction catalyzed by catalase. In the presence of ammonium ion, the formaldehyde reacts with acetylacetone, forming a yellow lutidine dye. The concentration of the lutidine dye formed is stoichiometric with the amount of cholesterol and is measured by the increase in absorbance at 405 nm. Optimization studies for each of the assay components of the method have been performed (18, 22, 23).

The cholesterol contents of 12 egg yolk samples determined by the enzymatic and GLC methods are presented in Table 1. The results obtained by the 2 methods were in excellent agreement ($P < 0.05$). Linear regression analysis (X-axis, data for the enzymatic method; Y-axis, data for the GLC method) gave a correlation of 0.963, with a slope of 1.05 and an intercept of −55.6 mg/100 g. Precision of the enzymatic method was excellent as indicated by the coefficient of variation (CV) obtained from 8 analyses

of each sample, which ranged from 1.0 to 3.4%. The mean cholesterol content of egg yolk determined by the enzymatic and GLC methods was 1237.2 and 1240.0 mg/100 g, respectively. Many data are available on the cholesterol content of egg yolk (9, 10, 26–29). Depending on the type of egg analyzed, the extraction method, and the determinative method used, a wide range of values, from 1200 to 2400 mg/100 g, have been reported. GLC methods are usually more accurate than either colorimetric or gravimetric procedures for determining the cholesterol content of egg yolk (9, 10) because of their greater specificity.

A recovery experiment was carried out by adding 500 μg cholesterol to 1 mL sample solution obtained from each egg yolk sample before enzymatic determination. Eight determinations were performed on each sample. A mean recovery of 97.4% with a standard deviation (SD) of 1.6% (CV 1.6) was obtained. Recovery studies of the methylene chloride–methanol method for extracting cholesterol from a variety of foods have been previously reported (24). In these studies, the average recovery was 99.1 ± 2.2% when cholesterol was gravimetrically determined.

The enzymatic method has been widely used in clinical laboratories for the determination of serum cholesterol because of its simplicity, reliability, and relatively high specificity (16–21). It has proven to be useful in the determination of milk (22) and tissue (23) cholesterol as well. Data obtained in the present study demonstrate that the enzymatic method is suitable for the quantitative determination of cholesterol in egg yolk and provides results comparable to those obtained by the GLC method. Whether this method can be applied to the determination of cholesterol in other foods requires further investigation.

REFERENCES

(1) *Code of Federal Regulations* (1980) Title 21, Chapt. 1, Subchapt. B, U.S. Government Printing Office, Washington, DC, Part 101, secs. 101.25 and 101.9

(2) Brown, H. B. (1968) *J. Am. Diet. Assoc.* **52,** 279–287

(3) Mann, G. V. (1977) *N. Engl. J. Med.* **297,** 644–650

(4) McGill, H. C. (1979) *Am. J. Clin. Nutr.* **12,** 2632–2636

(5) McNutt, K. (1980) *Nutr. Rev.* **38,** 353–360

(6) Williams, R. R., et al. (1981) *J. Am. Med. Assoc.* **245,** 247–252

(7) Shekelle, R. B., et al. (1981) *N. Engl. J. Med.* **304,** 65–70.

(8) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs. **17.017–17.022**

(9) Sweeney, J., & Weihrauch, J. L. (1976) *Crit. Rev. Food Sci. Nutr.* **8,** 131–159

(10) Tu, C., Powrie, W. D., & Fennema, O. (1970) *J. Food Sci.* **35,** 601–605

(11) Punwar, J. K. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 46–50

(12) Sheppard, A. J., Newkirk, D. R., Hubbard, W. D., & Osgood, T. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1302–1306

(13) Kovacs, M. I. P., Anderson, W. E., & Ackman, R. G. (1979) *J. Food Sci.* **44,** 1299–1301

(14) *Official Method of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs. **28.099–28.106**

(15) Newkirk, D. R., & Sheppard, A. J. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 54–57

(16) Flegg, H. M. (1973) *Ann. Clin. Biochem.* **10,** 79–83

(17) Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W., & Fu, P. C. (1974) *Clin. Chem.* **20,** 470–475

(18) Roschlau, P., Bernt, E., & Gruber, W. (1974) *Z. Klin. Chem. Klin. Biochem.* **12,** 403–407

(19) Deacon, A. C., & Dawson, J. G. (1979) *Clin. Chem.* **25,** 976–984

(20) Haeckel, R., Sountag, O., Rulpmann, W. R., & Feldmann, U. (1979) *J. Clin. Chem. Clin. Biochem.* **17,** 533–563

(21) Zak, B. (1980) *Lipids* **15,** 698–704

(22) Gentner, R. P., & Hassemann, A. (1979) *Milchwissenschaft* **34,** 344–346

(23) De Hoff, J. L., Davidson, L. M., & Kritchevsky, D. (1978) *Clin. Chem.* **24,** 433–435

(24) Chen, I. S., Shen, C-S. J., & Sheppard, A. J. (1981) *J. Am. Oil. Chem. Soc.* **58,** 599–601

(25) Sheppard, A. J., Hubbard, W. D., & Prosser, A. R. (1974) Interim Methodology Instructions No. 2, with Modification No. 1, Food and Drug Administration, Washington, DC

(26) *Composition of Foods* (1963) Agriculture Handbook No. 8, Agricultural Research Service, U.S. Department of Agriculture, Washington, DC

(27) Feeley, H., & Brown, M. L. (1972) *J. Am. Diet. Assoc.* **61,** 134–149

(28) Cunningham, D. L., Krueger, W. F., Fanguy, R. C., & Bradley, J. W. (1974) *Poult. Sci.* **53,** 384–391

(29) Bitman, J., & Wood, D. L. (1980) *Poult. Sci.* **59,** 2014–2023

Analysis of Fat-Soluble Vitamins. XXVI. High Performance Liquid Chromatographic Determination of Vitamin D in Fortified Milk and Milkpowder[1]

BEN BORSJE, ELLEN J. DE VRIES, JAKOB ZEEMAN, and FRITS J. MULDER
Duphar B. V., Research Laboratories, Weesp, The Netherlands

Vitamin D is determined by high performance liquid chromatography (HPLC) in samples containing other fat-soluble vitamins. The vitamin D in the unsaponifiable residue is extracted and separated from interferences by straight phase chromatography, and the fraction corresponding to vitamin D_3 is collected and quantitated using the AOAC official final action HPLC method for vitamin D_3. Analysis of a synthetic mixture gave reasonable recoveries. The method measures potential vitamin D_3 content in milkpowder samples containing 2 IU vitamin D/g in the presence of all known vitamin D_3 isomers, vitamin A, and vitamin E.

The determination of vitamin D at low levels, such as the levels in fortified milk, 35–50 IU/100 mL, is a time-consuming and tedious procedure. Several methods have been devised previously (1–4), each having its limitations and drawbacks. Particular attention has been given to the gas-liquid chromatographic determination of vitamin D in milk, but precollaborative testing proved unsatisfactory.

However, general interest in the determination of vitamin D in milk is increasing. For example, the Associate Referee on vitamin D was requested by the Northern Dairy Practices Council (5) to study the Henderson method (4) for its suitability as an official method, but a particular problem is encountered with previtamin D formation, a property of vitamin D. Verification of the Henderson method (4) resulted in recoveries of vitamin D of 43 and 68%. Study of the chromatographic system showed a peak with the same retention time as vitamin D to be a vitamin A isomer. The Thompson method (3) depends on labeled vitamin D to verify column performance.

An analytical procedure for determining vitamin D can be considerably shortened by making certain assumptions. First, potency, which is usually expressed in International Units in relation to bioactivity, is the sum of the biopotencies of both previtamin D and vitamin D (6, 7). This potency is determined by a physical method which separates vitamin D from previtamin D (8). However, previtamin D in low potency samples is difficult to separate from impurities; therefore, potency must be derived from the vitamin D peak only, allowing a correction for previtamin D. This correction can be calculated, dependent on the selected procedure, time, and temperature. Corrections under practical conditions will be about +20% of the vitamin D potency.

The second assumption concerns the quality of vitamin D used for milk fortification. In Europe, milk is enriched mainly by adding pharmaceutical grade vitamin D_3 (cholecalciferol). By definition, no inactive vitamin D isomers are present, as would be expected in mixtures of vitamin D isomers, so-called vitamin D resins, or vitamin D resin-containing products.

In the United States, however, no conditions are given for vitamin D in milk fortification, so vitamin D must be separated from its inactive isomers, such as tachysterol, trans-vitamin D (9), isotachysterol, and luministerol as present in irradiated 7-dehydrocholesterol or irradiated ergosterol.

From previous high performance liquid chromatographic (HPLC) work for the determination of vitamin D in concentrates and multivitamins (10–13), we learned that an analytical column is needed to separate vitamin D from its isomers and that a cleanup column is required to isolate the vitamin D family in one peak from the other fat-soluble vitamins and impurities. Different types of chemically bonded stationary phases (C8, C18, etc.) were examined and optimal results were obtained with a nitrile stationary phase in the straight phase mode. We had succeeded in using the standardizing analytical column (adopted in the AOAC HPLC method for vitamin D) both for concentrates and multivitamins, so it was collaboratively studied twice. In this study, we maintained this technique, and studied the cleanup procedure and isolation of

[1] For Part XXV, see de Vries, E. J., Mulder, F. J., & Borsje, B. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 61–70.
This report of the Associate Referee, F. J. Mulder (present Associate Referee, E. J. de Vries), was presented at the 93rd Annual Meeting of the AOAC, Oct. 15–18, 1979, at Washington, DC.

0004-5756/82/6505-1225–03$01.00

Table 1. Recovery of vitamin D at 2 levels from skimmed milkpowder samples (2 and 5 IU/g) as determined with external standard (cholecalciferol)

	2 IU/g samples			5 IU/g samples	
Day	B1	B2	B3	C1	C2
1	122.9[a]	109.4[a]	61.1[b]	100.1	90.9
2	98.5	92.9	95.6	95.9	90.1
3	105.1	107.8	101.1	91.3	95.1
4	109.5	108.0	111.9	101.9	102.2
5	113.2	115.4	114.0	106.8	104.7

Mean of Sample B	106.1%	Mean of Sample C	97.9%
Repeatability SD	2.5%	Repeatability SD	3.8%
Reproducibility SD	8.3%	Reproducibility SD	6.3%

[a] These recoveries were eliminated.
[b] Eliminated by the Dixon test.

Table 2. Recovery of vitamin D at 2 levels from skimmed milkpowder samples (2 and 5 IU/g) as determined with internal standard (Δ 4,6-cholestadienol)

	2 IU/g samples			5 IU/g samples	
Day	B1	B2	B3	C1	C2
1	110.8[a]	109.3[a]	62.9[b]	92.6	95.3
2	93.3	95.7	96.7	95.8	100.5
3	109.3	100.8	105.7	89.7	92.6
4	105.6	106.8	107.7	99.1	96.7
5	104.7	105.9	111.8	101.9	105.7

Mean of Sample B	103.6%	Mean of Sample C	97.0%
Repeatability SD	3.0%	Repeatability SD	2.4%
Reproducibility SD	6.1%	Reproducibility SD	5.1%

[a] These recoveries were eliminated.
[b] Eliminated by the Dixon test.

the unsaponifiable residue for vitamin D from milk.

METHOD

See part XXVII, E. J. de Vries & B. Borsje (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1228–1224.

Results and Discussion

Milk contains about 10% dry substances and the content of vitamin D in fortified milk is about 200 IU/L (2 IU vitamin D/g in the dried sample). To check the proposed HPLC method, we took unfortified, skimmed milkpowder containing no vitamin D_3 and mixed it with a known amount (2 IU) of vitamin D_3/g and 20 IU vitamin A/g. A second powder sample was mixed with a known amount (5 IU) of vitamin D_3/g and 50 IU vitamin A/g. Two levels of vitamin D are required to verify whether a systematical error is present.

We also studied the use of internal and external standards.

At these low levels of fortification in milkpowder, it is impossible to determine the previtamin D peak. A 45 min saponification at 80°C provides a constant ratio of vitamin D/previtamin D, from which the vitamin D potency content can be calculated using a factor. A cholecalciferol standard subjected to the entire procedure gave 80% cholecalciferol on analysis. Therefore, results of the HPLC method should be multiplied by the factor 1.25 to determine vitamin D potency.

Blank skimmed milkpowder containing only 50 IU vitamin A/g was studied to determine interfering substances, calculated as vitamin D in the vitamin D_3 fraction. This blank sample (containing no vitamin D) showed ≤0.2 IU cholecalciferol/g.

Two samples containing, respectively, 2 IU vitamin D with 20 IU vitamin A and 5 IU vitamin D with 50 IU vitamin A were analyzed daily in random sequence (Tables 1 and 2). Recoveries were determined with the internal standard (Δ 4,6-cholestadienol) and an external standard. For the statistical analysis, one determination in Sample B on the first day was eliminated (according to the Dixon test) as a significant outlier. We also eliminated (14), for simplifying the calculation, the replicates of Sample B from that day in Tables 1 and 2. Recoveries from Samples B and C are, respectively, 84.9% and 78.3% for external standard calculation, and 82.9% and 77.6% for internal standard calculation. The differences in recovery between Sample B (2 IU vitamin D/g) and Sample C (5 IU vitamin D/g) in Tables 1 and 2 are nearly significant. Apparently, recoveries at lower concentrations are more influenced by interfering substances.

For potential vitamin D content, all recoveries must be multiplied with a factor to be determined at the same time. There is no significant difference between calculations using an internal standard or an external standard. The internal standard procedure gives a nonsignificant better reproducibility of the standard deviation; however, the external standard is generally available and is therefore preferred. Tests with whole milkpowder gave similar results.

Recommendation

It is recommended that the proposed HPLC method be collaboratively studied for fortified skimmed milkpowder, milkpowder, and fortified milk.

Acknowledgments

We thank P. Hooghamer (Analytical Department, Duphar, B.V., Weesp) for his part in the experimental work, and P. van Bemmel (Statistical Department, Duphar B.V., Weesp) for the design of the statistical analysis.

References

(1) Panalaks, T. (1971) *J. Assoc. Off. Anal. Chem.* **54,** 1299–1303
(2) Bell, J. G., & Christie, A. A. (1974) *Analyst* **99,** 385–396
(3) Thompson, N. J., Maxwell, W. B., & L'Abbé, M. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 998–1002
(4) Henderson, S. K., & Wickroski, A. F. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1130–1134
(5) Northeast Dairy Practices Council, Minutes and Resolution of Business Meeting, November 3, 1978, Ellenville, NY
(6) Mulder, F. J., & van Strik, R. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 117–121
(7) Keverling Buisman, J. A., Hanewald, K. H., Mulder, F. J., Roborgh, J. R., & Keuning, K. J. (1968) *J. Pharm. Sci.* **57,** 1326–1329
(8) de Vries, E. J., Zeeman, J., Esser, R. J. E., Borsje, B., & Mulder, F. J. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 129–135
(9) Borsje, B., Heyting, A., Roborgh, J. R., Ross, D. B., & Shillam, K. W. G. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1003–1009
(10) Mulder, F. J., de Vries, E. J., & Borsje, B. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 1031–1040
(11) de Vries, E. J., Zeeman, J., Esser, R. J. E., Borsje, B., & Mulder, F. J. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 1285–1291
(12) de Vries, E. J., Mulder, F. J., & Borsje, B. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 61–70
(13) Mulder, F. J., de Vries, E. J., & Borsje, B. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 58–60
(14) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA

Analysis of Fat-Soluble Vitamins. XXVII. High Performance Liquid Chromatographic and Gas-Liquid Chromatographic Determination of Vitamin D in Fortified Milk and Milkpowder: Collaborative Study[1]

ELLEN J. DE VRIES and BEN BORSJE
Duphar B. V., Research Laboratories, Weesp, The Netherlands

Collaborators: L. Ameika; N. T. Baillies; N. Delano; E. J. de Vries; W. Egtberts; G. A. Fischer; E. George, Jr; P. J. Gillingham; W. Hancock; H. Knapstein; A. Monard; J. F. Muniz; G. Nedelkovitch; G. Nicolaux; E. Oelrich; G. F. Phillips; R. Pollman; E. F. Richter; H. Scholz; P. Schwarze; H. Steuerle; U. Thiele; V. A. Thorpe; C. Weaver; H. M. Wehr

A collaborative study of the high performance liquid chromatographic (HPLC) method for vitamin D in fortified milkpowder (skimmed and whole milk) and a milkpowder preparation was carried out on 182 samples distributed to 26 laboratories. Thirteen laboratories submitted results. The level of vitamin D was 2-7 IU vitamin D_3/g milkpowder. All samples also contained vitamin A. Three laboratories were excluded from the statistical evaluation because of incomplete results or deviation from the analytical procedure. Other laboratories reported acceptable results. At the same time, 63 samples were distributed to 9 laboratories which used a gas-liquid chromatographic method for determining vitamin D in milkpowder. Only one laboratory reported results. The HPLC method has been adopted official first action.

The high performance liquid chromatographic (HPLC) method for determining vitamin D[2] in fortified milk and milkpowder, presented in 1979 (1), was recommended for collaborative study. A vitamin D roundtable discussion was organized during the 93rd AOAC meeting to invite and instruct future collaborators for the study. At that time, it was requested that for those laboratories which have gas chromatographic equipment only, a parallel study be conducted using an unpublished gas-liquid chromatographic (GLC) procedure (2) on the same samples. A comparison with the AOAC biological method (3) for vitamin D was considered superfluous, because this HPLC method was tested on synthetic mixtures with a known amount of vitamin D, so the systematic error could be calculated.

The HPLC method is based on saponification and extraction of the unsaponifiable residue followed by an HPLC cleanup on a nitrile column to separate vitamin D and its isomers from interfering substances; the fraction corresponding to vitamin D is collected and quantitated using the AOAC HPLC method for vitamin D (4). The GLC method is based on saponification and extraction of the unsaponifiable residue followed by a digitonin precipitation of cholesterol, cleanup by Florisil chromatography, and, after silylation, GLC quantitation of the pyrocalciferols.

Collaborative Study

Samples

In this study, 5 fortified milkpowder preparations of different composition (Table 1) were assayed. Three samples contained pure cholecalciferol, in coated form (microbeadlets). Samples 1 and 2 were based on skimmed milkpowder, whereas Sample 3 was based on whole milkpowder. The vitamin D_3 content of these samples was calculated on a weight basis: Single determinations were carried out in these coded samples. Preparations 4 (containing vitamin D_3 resin) and 5 (containing cholecalciferol) were commercially available fortified milkpowder preparations, feed and food grade, respectively, from different manufacturers. From each of the Preparations 4 and 5, 2 differently coded samples were assayed, thus yielding independent duplicate determinations for each of these preparations.

Collaborators

For this collaborative study, we distributed 245 samples to 26 collaborators (HPLC) and 9 col-

[1] For part XXVII, see Borsje, B., de Vries, E. J., Zeeman, J., & Mulder, F. J. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1225-1227.
This report of the Associate Referee, E. J. de Vries, was presented at the 94th Annual Meeting of the AOAC, Oct. 20-23, 1980, at Washington, DC.
The recommendation of the Associate Referee was approved by the General Referee and Committee D and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1981) **64**, 432.

[2] Vitamin D is used here as a generic term for vitamin D_2 (ergocalciferol) or D_3 (cholecalciferol); previtamin D is used for previtamin D_2 or D_3.

0004-5756/82/6505-1228-07$01.00

Table 1. Composition of collaborative samples

Sample	Vit. D_3, IU/g	Vit. A, IU/g	Preparation
1	exactly 2.0	± 20	skimmed milkpowder
2	exactly 5.0	± 50	skimmed milkpowder
3	exactly 5.0	± 50	whole milkpowder
4	declared 7.6	±112	skimmed milkpowder[a]
5	declared 5.0	± 50	skimmed milkpowder[b]

[a] Dogfood preparation, so-called "bitch milk," also containing vitamins B_1, B_2, niacin, pantothenic acid, choline chloride, and biotin and trace elements.

[b] Fortified skimmed milkpowder, for the World Food Program, containing 5000 IU vitamin A and 500 IU vitamin D_3/100 g.

laborators (GLC). The HPLC collaborators were asked to test their equipment for separation of trans-vitamin D/previtamin D on the analytical column. If the peak resolution was better than 1, the column performance was satisfactory. The GLC collaborators were asked to (a) determine the presence of possible interfering materials in Florisil; (b) verify elution patterns on Florisil; (c) determine recoveries of spiked samples. If the recovery exceeded 85%, the column performance was satisfactory.

METHODS

Gas-Liquid Chromatographic Method

See ref. 2.

Vitamin D in Fortified Milk and Milkpowder

High Performance (Pressure) Liquid Chromatographic Method

Official First Action

(Applicable to fluid milk and milkpowder contg ≥1 IU vitamin D/g)
(*Caution:* See **51.039**)

43.B09 ***Principle***

Samples are saponified and extd. Vitamin D and isomers are sepd from interfering substances on cleanup column. Second column seps vitamin D from impurities. Vitamin D is corrected for amt previtamin D formed during saponification. Vitamin D is sum of vitamin D and previtamin D.

43.B10 ***Apparatus***

(a) *Liquid chromatograph.*—Hewlett Packard 1010A, or equiv., with 254 nm UV detector with 2 columns: cleanup and anal.

(b) *Cleanup column.*—Stainless steel, 250 × 4.6 (id) mm, packed with 10 μm particle size Sil60D-10CN. Typical operating conditions: chart speed, 1 cm/min; eluant flow rate, 1 mL/min; detector sensitivity, 0.128 AUFS; temp., ambient; valve injection vol., 200 μL; mobile phase, *n*-hexane contg 0.35% *n*-amyl alcohol.

(c) *Analytical column.*—Stainless steel, 250 × 4.6 (id) mm, packed with 5 μm particle size Partisil, passing system suitability test. Typical operating conditions: chart speed, 1 cm/min; eluant flow rate, 2.5 mL/min; detector sensitivity, 0.008 AUFS; temp., ambient; valve injection vol., 500 μL; mobile phase, *n*-hexane contg 0.35% *n*-amyl alcohol.

43.B11 ***Reagents***

(a) *n-Hexane.*—See **43.081(a).**

(b) *Antioxidant soln.*—0.1% butylated hydroxytoluene (BHT) in *n*-hexane.

(c) *Vitamin D std soln.*—USP Ref. Std Ergocalciferol (if sample labeled as contg vitamin D_2) or Cholecalciferol (if labeled as contg vitamin D or D_3). Accurately weigh ca 12.5 mg vitamin D std in 100 mL amber vol. flask. Dissolve without heat in toluene and dil. to vol. with toluene. Dil. 10 mL of this soln to 100 mL with mobile phase. Dil. 25 mL of this soln to 100 mL with toluene-mobile phase (5 + 95) for vitamin D std (3.125 μg/mL, 125 IU/mL). Prep. fresh daily.

(d) *System suitability std soln.*—Prep. soln contg 2 mg vitamin D_3 and 0.2 mg trans-vitamin D_3/g in vegetable oil. Peaks of trans-vitamin D_3 and previtamin D_3 must have ca same peak hts. If necessary, increase previtamin D_3 content by warming oil soln at 90° ca 45 min. Store soln at 5°.

43.B12 ***System Suitability Test for Analytical Column***

Dissolve 0.1 g system suitability std soln in 100 mL toluene-mobile phase (5 + 95) and chromatograph 200 μL. Det. peak resolution between previtamin D_3 and trans-vitamin D_3 as: $R = 2D/(B + C)$: where D = distance between peak max. of previtamin D_3 and trans-vitamin D_3; B = peak width of previtamin D_3; and C = peak width of trans-vitamin D_3. Performance is satisfactory if $R \geq 1.0$.

43.B13 ***Calibration***

Inject 200 μL vitamin D std soln onto cleanup column thru sampling valve, and proceed as in *Determination*, "... adjust operating conditions ..." Det. retention time of vitamin D on anal. and cleanup columns and peak ht of vitamin D

on anal. column. Retention time of vitamin D on cleanup column should be between 10 and 20 min; adjust amyl alcohol content of mobile phase, if necessary to achieve this situation.

43.B14 *Preparation of Sample*

(a) *Isolation of unsaponifiable matter from powder.*—Accurately weigh ca 50 g milkpowder into saponification flask. Add 100 mL alcohol, 25 mL 25% aq. Na ascorbate soln, and 25 mL 50% (w/w) aq. KOH soln. Reflux 45 min on steam bath. Cool rapidly under running H_2O. Transfer liq to separator with two 75 mL portions H_2O, two 25 mL portions alcohol, and two 100 mL portions ether. Shake vigorously 30 s and let stand until both layers are clear. Transfer aq. phase to second separator and shake with mixt. of 25 mL alcohol and 100 mL pentane. Let sep., and transfer aq. phase to third separator and pentane phase to first separator, washing second separator with two 10 mL portions pentane, adding washings to first separator. Shake aq. phase with 100 mL pentane and 25 mL alcohol and add pentane to first separator. Wash combined pentane exts with three 100 mL portions freshly prepd 3% soln of KOH in 10% alcohol, shaking vigorously. Then wash with 100 mL portions H_2O until last washing is neutral to phthln. Drain last few drops of H_2O, add 4 sheets 9 cm filter paper in strips to separator, and shake.

Transfer dried pentane ext to 500 mL r-b flask, rinsing separator and paper with pentane, and add 1 mL antioxidant soln. Evap. to dryness under vac. by swirling in H_2O bath at $\leq 40°$. Cool under running H_2O and restore atm. pressure with N. Dissolve residue immediately in 2–3 mL toluene-mobile phase (5 + 95).

Transfer ext to 10 mL r-b flask, rinsing 500 mL r-b flask with pentane. Evap. under N stream at room temp. Dissolve residue immediately in 2.0 mL toluene-mobile phase (5 + 95). Use this soln as working sample soln for injection.

(b) *Isolation of unsaponifiable matter from fluid milk.*—Pipet 200 mL milk into 1 L saponification flask. Add 300 mL alcohol, 5 g Na ascorbate, and 50 mL 50% aq. KOH. Proceed as in (a), beginning, "Reflux 45 min . . ."

43.B15 *Determination*

(a) *Cleanup.*—Inject 200 μL working sample soln onto cleanup column thru sampling valve and adjust operating conditions of detector to give largest possible on-scale peaks for vitamin D. Collect fraction between 2 min before and 2 min after vitamin D peak, in 10 mL vol. flask. Add 1 mL antioxidant soln and evap. to dryness under N stream. Dissolve residue immediately in 2 mL toluene-*n*-hexane (5 + 95). Use this soln for injection onto anal. column.

Note: To regenerate cleanup column, wash with amyl alcohol-*n*-hexane (10 + 90) at 6 mL/min until detector response returns to 0 (ca 15 min). Switch to mobile phase, hexane contg 0.35% amyl alcohol. Column is ready for reuse.

(b) *Assay.*—Inject 500 μL soln, (a), onto anal. column thru sampling valve, and adjust operating conditions of detector to give largest possible on-scale peaks of vitamin D. Repeat injections of sample and stds to verify that response remains const. Measure peak ht of vitamin D in sample and external std solns.

(c) *Calculation.*—Vitamin D potency, IU/g = $(1.25 \times P \times W' \times V \times 40\,000)/(P' \times W \times V')$ where 1.25 = correction factor for previtamin D formed during refluxing for saponification; P and P' = peak ht of vitamin D in sample and ref. std, resp.; W and W' = g sample and mg ref. std, resp.; V and V' = total mL sample and ref. std solns, resp.; 40 000 = IU vitamin D/mg USP ref. std.

Results

Tables 2 and 3 summarize the various columns and packings used and the retention times and resolutions obtained in the collaborative study. Eight collaborators gave their comments. Two collaborators stated that the method is time consuming. Four collaborators encountered some problems with the saponification step, which they solved themselves by increasing the amount of "saponification liquid" and/or by using a magnetic stirrer with heating plate. One collaborator had difficulty in obtaining the cleanup column. Moreover, he indicated that this cleanup column showed poor separation and recommended that this method be further studied with possible modifications. Another collaborator stated that the cleanup step appeared to work well, but a detector with a high noise level created problems with the analytical column. One collaborator suggested regenerating the cleanup column by injection of approximately 2 mL (5 × 400 μL) of 10% *n*-amyl alcohol in *n*-hexane, instead of washing the column with the same mixture. Some collaborators had no comments, or stated that the method works well and may be a sound basis for the development of the HPLC assay of vitamin D in foods and feeds.

The collaborative results are given in Table 4.

Table 2. Columns and packing used for cleanup column

Lab.	Column, cm × mm	Packing	Particle size, μm	Mobile phase, % amyl alcohol	Flow rate, mL/min	Ret. time, min
1	30 × 3.9	μBondapak CN	10	0.35	1.5	7
2	25 × 4.6	Partisil PAC	10	0.7	1	34
3	25 × 2.6	Spherisorb Nitrile	5	0.5	1.5	18
4	25 × 4.6	R-Sil CN	10	1.6	1.0	14
5	25 × 4.6	Spherisorb CN	5	0.35	1.5	19–20
6	25 × 4.0	Lichrosorb CN	10	0.35	1.5	9
7	25 × 4.6	SIL-60D-10 CN	10	0.35	1	22
8	30 × 4.0	μBondapak CN	10	0.35	3.0	5
9	25 × 4.6	SIL-60D-10 CN	10	0.35	1.0	12
10	30 × 3.9	SiOH	10	0.53	2.0	14.4
11	22 × 4.0	Polygosil 60-10 CN	10	0.35	2	11
12	30 × 3.9	μBondapak CN	10	0.35	1.0	8
13	25 × 4.6	SIL-60D-10 CN	10	0.35	1	—
13A	25 × 4.6	μBondapak C_{18}	—	—[a]	1	—

[a] Acetonitrile–methanol–water (50 + 50 + 5).

Fourteen laboratories participated in this study, of which 10 performed the method according to the instructions. Laboratory 8 used a C_{18} instead of a silica column in the analytical procedure. Laboratory 10 used a silica column in the cleanup procedure instead of the prescribed one. Laboratories 8 and 10 reported an incomplete set of results and were excluded from the statistical evaluation. Laboratory 12 analyzed Sample 4 biologically by the double level rat bioassay and reported under number 12A. Laboratory 13, meeting difficulties with the extraction procedure, modified the procedure substantially, by replacing the isolation procedure by his own laboratory method. Furthermore, he applied his own laboratory method for vitamin D in feeds to the samples and reported these results under 13A. Laboratory 14 analyzed the samples by using the GLC method of Muniz and Wehr (2).

Statistical Evaluation

Table 1 gives the composition of the different preparations; Samples 1 and 2 were identical except for the difference in vitamin D and vitamin A levels. For the simplest statistical analysis, equal numbers of replicate determinations per sample must be available. In this context, single determinations of different samples, which only differ in vitamin D content, may be considered as replicates.

Hence, for purposes of simplicity, the statistical analysis, according to Steiner (5) is, in the first instance, confined to the results of Samples 1 + 2, 4, and 5. To detect laboratories which show consistently high or low values, the ranking test described by Youden (5) was applied to sums of paired observations. The pairs are Samples 1 and 2, and the duplicates of Samples 4 and 5. No

Table 3. Columns, packings, and resolution obtained with analytical column

Lab.	Column, cm × mm	Packing	Particle size, μm	Mobile phase, % amyl alcohol	Flow rate, mL/min	Ret. time, min	Resolution, pre/trans
1	25 × 4.6	Partisil	5	0.35	3	25	3
2	25 × 4.6	Partisil	5	0.35	2.5	26.4	1.7
3	25 × 4.0	Lichrosorb SI 60	5	0.5	2.5	17	2.5
4	25 × 4.6	R-Sil	5	0.6	2.5	17	1.66
5	25 × 4.6	Partisil	5	0.35	2.5	26.5	1.7
6	25 × 4.0	Lichrosorb SI 60	5	0.35	1.9	29	1.85
7	25 × 4.6	Partisil	5	0.35	2.5	27	1.14
8	30 × 4.0	μBondapak C_{18}	10	0.35	2.5	9.5	≥1
9	25 × 4.6	Partisil	5	0.35	2.5	14	1.8
10	30 × 3.9	SiOH	10	0.53	2.0	14.4	—
11	22 × 4.0	Partisil	5	1.0	2	13	2
12	30 × 4.6	μPorasil	10	0.35	2.5	18	—
13	20 × 4.6	Partisil	5	0.7	2.5	10	—
13A	20 × 4.6	Partisil	5	1.0	2.5	10	—

Table 4. Collaborative results (IU/g) for determination of vitamin D_3 in milkpowder [a]

Lab.	Sample 1	2	3	4	5
1	0.54	4.5	10.3[b]	4.4	3.5
				4.6	2.5
2	0[c]	5.70	5.70	5.19	3.67
				6.20	5.36
3	3.92	8.16	6.40	6.22	5.66
				8.13	5.45
4	4.3	6.5	6.5	4.8	5.1
				6.2	5.7
5	3.4	6.3	7.4	5.9	5.4
				7.8	4.6
6	3.3	6.3	6.0	7.3	5.5
				7.0	7.0
7	4.0	6.3	7.0	4.2	5.4
				3.7	9.1
8[b]	4.24	10.04	—	—	16.47
				7.83	8.10
9	2.6	6.2	5.7	8.1	5.1
				8.5	6.0
10[b]	3.2	6.6	9.98	70.3	9.2
				—	15.3
11	2.6	8.2	5.4	6.9	5.0
				19.0[b]	6.1
12	1.49	7.72	3.01[b]	3.22	7.98
				3.56	4.14
12A				7.6[d]	
13[b]	1.8	2.3	4.3	2.2	3.4
				2.4	3.2
13A[b]	2.0	6.4	2.0	4.5	6.2
				5.5	5.0
14[e]	2.5	6.0	4.2	4.5	7.7
				5.6	2.5
Mean	2.62	6.59	6.26	5.89	5.41

[a] See text for description of methods.
[b] Excluded from statistical analysis; see text.
[c] Not detectable.
[d] Double level rat assay; see text.
[e] GLC results; see text.

outlying laboratories were found at the 5% level of significance.

Next, the data were examined for incidental outlying results per sample by means of Dixon's test on sums of paired observations, and were significant (at the 5% level) on Sample 4 from Laboratory 11. Because only one of the duplicates is apparently wrong (poor saponification), it was decided to exclude that particular value (19 IU/g) from statistical analysis.

One of the requirements underlying the statistical analysis (analysis of variance) is homogeneity of variation between- and within-laboratories. The test described by Steiner (5) was performed in order to check these assumptions, and did not reveal heterogeneity at the 5% level of significance (Table 5).

The mean square for laboratory × sample interaction was compared with the replicates mean square by means of the F-test, and was significant at the 5% level, indicating that differences between laboratories are not the same for the 3 samples.

Table 5. Analysis of variance of collaborative results (IU/g) (Samples 1 + 2, 4, and 5) for HPLC determination of vitamin D in milkpowder

Source of variation	Degrees of freedom	Mean square
Laboratories (L)	9	5.1691
Samples (S)	2	9.1182
L × S interaction	18	2.4776
1 vs 2	1	79.2020
Replicates	28	1.2126

Estimate of repeatability standard deviation, S_0 = 1.10 IU/g (CV = 21%; df = 28)

Estimate of reproducibility standard deviation, S_x = 1.51 IU/g (CV = 28%; df[a] = 39)

[a] Approximated by Satterthwaite's formula (6).

Table 6. Means of 2 replicates of Samples 1 + 2, 4, and 5 and single determinations on Sample 3 (IU/g) for HPLC determination of vitamin D in milkpowder

	Sample			
Lab.[a]	1 + 2	3	4	5
1	2.52	10.3[b]	4.50	3.00
2	2.85	5.7	5.70	4.52
3	6.04	6.4	7.18	5.56
4	5.40	6.5	5.50	5.40
5	4.85	7.4	6.85	5.00
6	4.80	6.0	7.15	6.25
7	5.15	7.0	3.95	7.25
9	4.40	5.7	8.30	5.55
11	5.40	5.4	6.90	5.55
12	4.60	3.0[b]	3.39	6.06

[a] Data from Laboratories 8, 10, 12A, 13, 13A, and 14 excluded from statistical analysis; see text.
[b] Rejected by Dixon's test at the 5% level.

The bias of the method for Sample 1 can be obtained, because the difference in vitamin D content between Samples 1 and 2 is accurately known (3.000 IU/g). The estimate of the difference is 3.97 IU/g. Hence the bias with 95% confidence limits is estimated by

$$\text{mean bias} = 0.97\ (-0.04 \text{ to } +1.97)\ \text{IU/g}$$

For statistical analysis of all samples, the unreplicated data on Sample 3 can be incorporated by analysis of variance of the means of Samples 1 + 2, 4, and 5, together with the single determinations on Sample 3 (see Table 6). The analysis is based on the following mathematical model:

$$y_{ij} = \mu + 1_i + S_j + X_{ij} + C_{ij} \qquad (1)$$

where μ = general mean; 1_i = stochastic (error) term for labs, $E(1_i) = 0$, $V(1_i) = \sigma_L^2$; S_j = component for samples $\sum_{j=1}^{4} S_j = 0$, j = 1, 2, 3, 4 samples; X_{ij} = stochastic interaction term, $E(X_{ij}) = 0$, $V(X_{ij}) = \sigma_x^2$; C_{ij} = stochastic (error) term within labs, $E(C_{ij}) = 0$, $V(C_{ij}) = \sigma_2^2$ for j = 1, 2, 3 = σ^2 for j = 4. The various stochastic terms can be considered as normally distributed and mutually uncorrelated.

The analysis of variance yields mean squares for laboratories and for samples and an interaction mean square (see Table 7). Using the postulated model, the expectations of the mean squares can be obtained after some straightforward but tedious algebra. These expectations are displayed in Table 7. The reproducibility standard deviation, $\sqrt{\sigma^2 + \sigma_x^2 + \sigma_L^2}$, can now be estimated by the following linear combination of mean squares:

Table 7. Analysis of variance of collaborative results (IU/g) of all samples for HPLC determination of vitamin D in milkpowder

Source of variation	Degrees of freedom	Mean square	Expectation of mean square
Laboratories (L)	9	3.0236	$\frac{5}{8}\sigma^2 + \sigma_x^2 + 4\sigma_L^2$
Samples (S)	3	3.9940	not relevant
L × S interaction	25	1.0779	$\frac{5}{8}\sigma^2 + \sigma_x^2$

Estimate of reproducibility standard deviation, S_x = 1.42 IU/g (CV = 26%; df[a] = 42)

[a] Approximated by Satterthwaite's formula (6).

$$S_x = \tfrac{1}{2}\sqrt{MS_1 + 3\,MS_2 + 1.5\,MS_3}, \qquad (2)$$

where MS_1 = mean square between labs (Table 7); MS_2 = interaction mean square (Table 7); MS_3 = replicates mean square (Table 5).

Before the analysis of variance was performed, the data in Table 6 were subjected to the ranking test for outlying laboratories; there was no statistical significance at the 5% level. Furthermore, Dixon's test was applied to the data of Sample 3. The result of Laboratory 1 for Sample 3 (10.3 IU/g) was significant at the 5% level. This result was regarded as an outlier and was omitted in the subsequent analysis of variance. The low result of Laboratory 12 (3.01 IU/g) was also significant, and this value was also excluded from statistical analysis. The resulting missing value was dealt with by the missing value technique. As a consequence, the expectation of the mean square between laboratories given in Table 7 must now be regarded as a close approximation. The estimate of the reproducibility standard deviation according to formula 2 is S_x = 1.42 IU/g (CV = 26%), in close agreement with the estimate based on the analysis of the data for Samples 1 + 2, 4, and 5 only. The estimate of the repeatability is, of course, the same by both analyses.

Discussion

The saponification and extraction procedure is a rather complicated method for instrument-minded people. Some practice is needed to use this procedure. Too gentle shaking in the extraction, and evaporation at an increased temperature, may give negative systematic errors in the range of 10–25%. Analysts inexperienced in the HPLC determination of vitamin D, especially at this low level, usually encounter many problems in the chemical part of the procedure, and attempts should be made to simplify the saponification.

The fact that the mean values for Samples 1

and 2 (skimmed milkpowder) exceed the level of the added amount of vitamin D should not be contributed to recovery only, but to the presence of various sterols in this natural nutrient. The biological result of 7.6 IU/g reported by Laboratory 12A confirms the declared potency.

Study of GLC procedure for vitamin D in milkpowder seems to be stalled: Of 9 collaborators, one gave no response, 2 did not report their results, another 2 had problems with the Florisil recoveries (40–60%), and one did not collaborate for other reasons. Four of the 9 collaborators are either interested in or converted to the HPLC procedure. One collaborator did report results (Laboratory 14, in Table 4). It is clear that no recommendation can be made on the GLC procedure.

Recommendations

It is recommended (*1*) that the HPLC method for the determination of vitamin D in fortified milk and milkpowder be adopted as official first action; (*2*) that the HPLC study be continued to simplify the method.

Acknowledgments

The Associate Referee thanks the following collaborators:

N. T. Baillies and E. F. Richter, Raltech Scientific Services, Madison, WI

N. Delano and L. Ameika, Diamond Shamrock Corp., Harrison, NJ

W. Egtberts, Duphar B. V., Weesp, The Netherlands

G. A. Fischer and C. Weaver, Borden Inc., Columbus, OH

E. George, Jr and R. Pollman, Dept of Agriculture, State Food Laboratory, Albany, NY

P. J. Gillingham, Peboc Ltd, Llangefni, UK

H. Knapstein and H. Scholz, Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Kiel, GFR

G. Nedelkovitch and A. Monard, Assoc. Pharm. Belg., Bruxelles, Belgium

G. Nicolaux, Societé A.E.C., Commentry, France

J. F. Muniz and H. M. Wehr, State Dept of Agriculture, Salem, OR

E. Oelrich and P. Schwarze, E. Merck A. G., Darmstadt, GFR

G. F. Phillips and W. Hancock, Laboratory of the Government Chemist, London, UK

U. Thiele and H. Steuerle, B.A.S.F., Ludwigshafen, GFR

V. A. Thorpe, State Dept of Agriculture, East Lansing, MI

We are grateful to the following companies for supplying materials used in the study: Borden Inc., Columbus, OH, and affiliates; Melkunie Gouda, The Netherlands; Melkfabriek Zevenbergsche Hoek, The Netherlands; Duphar B.V., Weesp, The Netherlands. We also thank P. van Bemmel (Statistical Department, Duphar B. V., Weesp, The Netherlands) for performing the statistical analysis. Furthermore, we gratefully thank Frits J. Mulder, consultant to Duphar B. V., for his stimulating interest and helpful suggestions for this study.

References

(1) Borsje, B., de Vries, E. J., Zeeman, J., & Mulder, F. J. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1225–1227

(2) Muniz, J., & Wehr, H. M. (1979) GLC Procedure for the Determination of Vitamin D_2 and D_3 in Milk, Oregon Department of Agriculture, Laboratory Services, Salem, OR (unpublished)

(3) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs. **43.195–43.208**

(4) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs. **43.079–43.086**

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

(6) Satterthwaite, F. E. (1946) *Biometric Bull.* **2**, 110–114

SYMPOSIUM ON COMPUTERS IN THE LABORATORY

This symposium was held on October 20, 1981, at the 95th Annual Meeting of the Association of Official Analytical Chemists. Nine papers were presented by speakers representing government, industry, and university laboratories. The purpose of the symposium was to present to the analytical chemist an overview of how the computer can be used as a tool or aid for many different applications in today's laboratory. The papers were designed to be tutorial in nature, or to present specific applications of the computer in the analytical laboratory.

The real impact of the computer and the microprocessor is now being felt and seen both in the instrumentation and in the way that analyses are performed in the laboratory. If today one purchases a mass spectrometer, Fourier transform (FT) nuclear magnetic resonance spectrometer, FT-infrared spectrometer, gas chromatograph, high pressure liquid chromatograph, scintillation counter, or any one of many instruments too numerous to list, one would find that each incorporates a complete minicomputer system and/or one or more microprocessors to run and control the instrument. The analyst can view the computer or microprocessor as merely one additional tool to be used to accomplish the required analytical task. We hope that these symposium papers will serve to illustrate why and how automation in the laboratory can be carried out successfully. Computers in the laboratory can free the analyst from tedious, repetitious, time consuming activities of the past, and increase his or her productivity.

LAURENCE R. DUSOLD
Symposium Chairman

Food and Drug Administration
Washington, DC 20204

Transfer of Reports from an Automated Gas Chromatographic System to a Large Computer for Final Processing

MARION CLOWER, JR
Food and Drug Administration, Division of Chemical Technology, Washington, DC 20204

An automated gas chromatographic system consisting of a Hewlett-Packard 5710 gas chromatograph, a Varian 8000 autosampler, and a Spectra-Physics 4000 laboratory data system has been interfaced to FDA's large, central computer facility by using a Tektronix 4923-01 digital cartridge tape recorder. The autosampler provides single or multiple injections of up to 60 samples. The data system is preprogrammed with appropriate sample and standard information in addition to parameters for proper peak integration. A chromatogram is traced and a report of integration area counts for each peak detected is prepared by the data system after each chromatographic analysis. A duplicate of each report is recorded on the data cartridge. After analysis of all samples, the quality of chromatography is examined for acceptability. The chromatographic reports contained on the data cartridge may then be entered into the computer via a terminal equipped with a similar digital cartridge tape recorder. Translation of the reports, correlation of responses from sample and standard solutions, all calculations, and a complete report of each sample analysis are obtained by using the computer programming language APL. The data system-cartridge tape recorder interface, as well as data reduction using the large computer, is discussed.

The Spectra-Physics 4000 laboratory data system (1) is a multichannel microprocessor-based data system specifically designed for use with gas and/or liquid chromatographs. The installation described here is used by Food and Drug Administration (FDA) analysts conducting research in the area of analytical methodology for pesticide residues in food and feeds. This system is used to process data from recovery studies in which residue identities are known, as well as in the analysis of samples containing unknown pesticide residues. In its current configuration, the system is interfaced with 1 liquid chromatograph equipped with a fluorescence detector and 5 gas chromatographs equipped with electron capture, electrolytic conductivity, or flame photometric detectors.

The system was purchased to increase reliability and efficiency in processing the relatively large amount of data obtained from overnight use of autosampler-equipped chromatographs. These goals were readily achieved once the analysts became familiar with the organization and operation of the data system.

The introduction to automated data processing provided through use of the system clearly demonstrated the benefits that automated data processing techniques offer to the laboratory analyst. The availability of the FDA's large central computer facility, the Parklawn Computer Center, suggested that minimal effort would be required for further application of automated data processing to pesticide residue analysis. Additional data reduction, advanced statistical analysis, graphic representation, and archival storage and retrieval of data would all be possible once the data were transferred to the computer system. Although manual entry into the computer of results of chromatographic analyses is possible, it is extremely time-consuming and error-prone. Direct transfer of data in digital form to a magnetic tape and subsequent entry into the computer from the tape eliminates these difficulties. At a minimum, the taped data provide a duplicate copy of analytical results and a highly compact form of data storage.

This paper describes the operational structure of the automated gas chromatographic system. An interface for addition of a digital cartridge tape recorder to the data system and the mechanics of transfer of chromatographic reports to the tape are presented in detail. Examples of computer processing of the data via user-written computer programs are given to illustrate current and potential use of the entire computer-assisted laboratory data processing system.

The Data System

The SP 4000 laboratory data system is a modular, multichannel data reduction system designed to monitor a chromatographic detector signal, integrate detected peaks, and prepare an analytical report of all information pertaining to the chromatographic analysis. Data from 16 chromatographs can be accommodated by the central processor unit (CPU), the hub of the system. The CPU contains memory for storage of operational parameters as well as for all cal-

Presented at the Symposium on Computers in the Laboratory, 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

0004-5756/82/6505-1236-13$01.00

culations required for data processing. Each chromatograph in the system is connected to a data interface module (DIM). All DIMs are linked to each other and to the CPU, thereby forming a communication network for the entire system. The DIM monitors and digitizes the output and then transmits this information to the CPU for processing. When the chromatographic run is completed, an analytical report is prepared by the CPU and sent back to the DIM. The report is then printed at a specified output device. In most cases, this is the printer-plotter connected to the DIM. The DIM also has control signals available for operation of autosampler and/or chromatograph relays. Hence, each DIM with its chromatograph and printer-plotter constitutes a single data acquisition unit (i.e., channel) within the data system.

Data acquisition and processing are controlled by SP 4000 parameter files. A file, composed of 6 sub-files, is set up for a given type of chromatographic analysis and controls peak integration sensitivity, baseline correction, and area allocation. Pertinent sample information, the desired post-run calculations, and the format of the report are also controlled by the file. Once a file has been created, it contains default values for all parameters necessary for use of the file in a simple chromatographic analysis. Most users of the SP 4000, however, prefer to tailor the file parameters to the specific task at hand. Although a file must be used to acquire and process data each time a sample is analyzed, in most cases a single file may be used repeatedly for any number of samples to be analyzed under identical chromatographic conditions. Addition of an autosampler to the chromatograph-SP 4000 system permits a large number of samples to be analyzed automatically without assistance by the analyst.

Operation of the system can be controlled by programs. This limited programmability is provided by sets of instructions, called procedures, which are executed by the CPU. Procedures are used to control the timing of data acquisition, data storage, report functions, and output of messages. Direct control over data acquisition through the ability to manipulate files and other procedures is also available. Under procedure control, for example, any file parameter can be automatically set to the value desired for the next analysis. The increased flexibility of automatic control of the SP 4000 provided by a procedure gives the analyst greater freedom of operation of the system in the unattended mode.

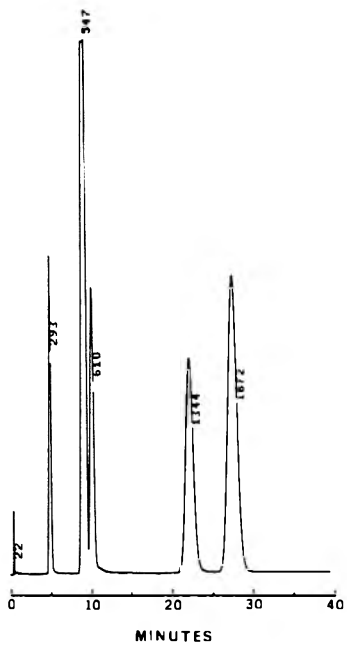


Figure 1. Typical SP 4000 chromatogram recorded by printer-plotter of solution containing (in order of elution) 4.91 ng parathion, 9.75 ng dieldrin, 1.85 ng endrin, 8.35 ng methoxychlor, and 7.4 ng mirex chromatographed on 5% OV-101 liquid phase on Chromosorb W(HP) with electron capture detection.

A procedure is composed of a series of steps, or directions. Each of the 8 types of steps available is named according to the function it performs: acquire, report, wait, message, command, jump, number, and halt. The order, number, and type of steps contained in a procedure is determined by the user and is based on the task to be accomplished by the procedure. A procedure may contain from 1 to 255 steps. Each step, regardless of overall function, is composed of eight 1-digit flag registers and nine 3-digit work registers. The flag registers turn on or off specific software switches that are related to the function of the step. The work registers contain numbers used by the CPU to define the precise manner of execution of the step. Two procedures for transfer of SP 4000 reports to a magnetic tape cartridge are included in the Appendixes. Application of these procedures is discussed in the section describing the interface between the SP 4000 and the tape unit.

The printer-plotter is a digital recorder that uses z-fold thermal paper. A 5×7 dot matrix is used for printing characters. An eighth element in the print head is used for tracing chromato-

FISH EXTRACT COMPARISON 1981 JUL 13 18:14:08

CHANNEL 5 RUN 2 FILE 5 METHOD 5

VIAL 2 TRAY 2

INDEX 4 SAMPLE 2-2 STD NFF *A

S.AMT=

NAME	CONC	RT	AREA	KF
PARATHION	15270	293	15266	1
DIELDRIN	132900	547	132940	1
ENDRIN	25790	610	25788	1
MOXYCHLOR	45750	1344	45753	1
MIREX	74760	1672	74759	1
TOTALS	294500		294506	

Figure 2. Typical SP 4000 report. Data correspond to chromatogram shown in Figure 1.

grams. A typical chromatogram recorded on a printer-plotter after injection of a solution of reference standards is given in Figure 1. Retention times (in seconds) are printed on the backside of each peak. Because peak area is being integrated by the system, off-scale peaks will not affect quantitation, provided that the response is within the linear range of the detector being used.

When the run is completed, data are retrieved from storage and calculations are completed as specified in the file. An analytical report, such as that shown in Figure 2, is then sent to the output device specified in the file used to collect the data. This may be any printer-plotter in the system, or some other output device such as a teletypewriter or tape recorder.

The report contains information from the chromatographic analysis and documentation of the SP 4000 routines used in processing the chromatographic data. The name of the file and time of injection are given in the first line of the report. Line 2 contains pertinent SP 4000 documentation. A listing of file 5, for example, will document all parameters that controlled integration of peaks during the run. Line 3 gives the position of the sample vial in the autosampler rack from which the injection was made. The name of this vial can be compared with the sample name entered into the file and printed on the next line of the report. If these names do not match, the wrong vial was injected or incorrect sample information was entered into the file. Therefore, any sample information used by the SP 4000 in these calculations will not necessarily correspond to the actual sample injected. To facilitate this comparison, the position of the sample vial in the autosampler tray has been included as part of the sample name which was entered into the file.

Data from peaks detected during the run are listed in the second half of the report. Peak retention time and raw area are printed in most report formats available. All peaks meeting detection criteria in the file are listed in order of increasing retention time and are assigned an identification number. In this report, all peaks fall within a retention window specified in the file Consequently, the peak identification numbers are replaced by the names corresponding to these retention windows. Values of unity were entered for all calculation factors so that the reported concentrations represent the raw area counts "rounded" to a maximum of 4 non-zero digits. The SP 4000 uses concentration values, not raw area counts, for any additional calculations it performs.

The chromatograph, of course, is the center of the instrumentation and the reason for the existence of the data system. The autosampler used with the chromatograph occupies a critical position within the entire system. The autosampler is the source of unattended operation for the chromatographic system. Its operation is independent of the SP 4000. The chromatographic system, controlled by the autosampler, and the SP 4000 are synchronized through the autosampler's internal clock. The autosampler's analysis time (between injections) is adjusted to include time for the chromatographic analysis and time for the SP 4000 to print the report. The autosampler signals the SP 4000 to begin the run at

the moment of injection. If this signal is received while a report is being printed, the run will not be started until after completion of the report. In this case the moment of injection for the SP 4000 (and all peak retention windows) will not correspond to the actual time of injections. Several extra minutes are usually added to the autosampler's analysis time to accommodate reports of longer than expected length. With proper synchronization of autosampler and SP 4000 operation, the injection-integration-report sequence will be repeated for each sample.

Digital Tape Unit Interface

Addition of a digital cartridge tape recorder to the SP 4000 provides a means of obtaining almost unlimited additional processing of the data contained in the analytical reports produced by the data system. The data cartridge unit becomes a component of the SP 4000 and appears to the CPU as another output device available to all users of the system. The CPU transmits the report to the output device specified by the file; the nature of that device is immaterial. After the report is printed at the printer-plotter containing the chromatogram, the file output device is changed. A second, duplicate report is then sent to the data cartridge unit. (File and procedure listings can also be output to the data cartridge.) Once on the data cartridge, the reports may be entered into a computer through a computer terminal interfaced with a similar digital data cartridge unit. A single data cartridge unit may be used if it is compatible with both the SP 4000 and the computer terminal. In this work, the computer extracts selected information from a series of reports. These data are then used to prepare comprehensive reports of sample analyses and pesticide residue quantitations as discussed below. A Tektronix Model 4923-01 digital data cartridge recorder (2) is an acceptable unit for this application. Digital data cartridges, certified 100% error-free on receipt according to American National Standards Institute specifications[1] are readily available for this type of data cartridge unit.

The interface between the data cartridge unit and the CPU of the SP 4000 is composed of an interface cable and a computer/teletype interface board. Both components are available from Spectra-Physics and can be installed by the user. The RS-232-C interface cable (part number A0640) connects the data cartridge unit to the interface board (part number A649-010) installed in the CPU. The CPU has a capacity for 2 interface boards, each of which may have 2 channels (output devices in this application). Only one channel in this system is currently in use. Transfer of a typical report (Figure 2) to the data cartridge requires less than 3 s at 2400 baud. This is very fast relative to printer-plotter speed (less than 100 baud), for which several minutes are required. Therefore, the data cartridge unit is rarely busy. For these reasons, it appears that only a very heavy user load or extremely large sample volumes from several users would require addition of another interface cable and data cartridge unit.

One modification must be made in the wiring of the interface board connector of the interface cable. In this installation the data cartridge unit is always ready to receive a signal and transmits no "clear to send" signal to the CPU. The CPU, however, must receive such a signal before it will transmit data. A wire installed between pins A4 and B2 of the interface board connector uses the +5V signal at pin A4 to continuously provide the required "clear to send" signal for the data cartridge unit. Table 1 contains a description of the wiring in the modified interface cable.

Successful data transfer requires that both transmitter and receiver use the same transfer rate and data format. The rate of data transfer for each channel is determined by 2 independent rotary switches. Transfer rates of 110, 300, 600, 1200, and 2400 baud are available. The fastest rate available that is also compatible with the data cartridge unit, 2400 baud, is selected. Five slide switches control parity bit (on), word length (8 bit), and stop bit (on). The interface board switches must be set to the proper positions before the board is installed in the CPU circuit board cage. The 2 channels on the board are given output device numbers based on the position of the channel slide switch.

The Tektronix 4923-01 functions as a data receiver only. The on-line switch must be off and the local-test-computer switch must be in the local position. The modem cable supplied with the unit is not used. For simplicity, both the receive and transmit rate switches are set to 2400 baud. The unit can function at data transfer rates of up to 9600 baud. To record data on a data cartridge, power is applied, a data cartridge is installed, and the unit is placed in the "write" mode. Data will be recorded when received. Once the chromatographic run is complete, the unit is stopped, the data cartridge is removed, and the power is turned off.

[1] For example, DC-300A certified; DC-300XL certified from 3M Company or equivalent.

Table 1. Wiring of modified Spectra-Physics RS-232-C interface table (A0640)

Interface board connector			Tape unit connector	
Pin	Purpose	Wire	Pin	Purpose
B1	ground	blue/orange	1,7	ground
B6	transmit	yellow	2	receive
B3	receive	green	3	transmit
B4	request to send	violet	4	request to send
B2[a]	clear to send	brown	5	clear to send
A4[a]	data terminal ready	red	20	data set ready

[a] SP cable modified by wiring +5V of pin A4 to pin B2 to provide "clear to send" signal to SP 4000 CPU.

Recording Reports on Tape

Normal use of the SP 4000 requires 3 steps for each sample to be analyzed: (1) injection of the sample into the chromatograph, (2) acquisition of integration data, and (3) processing and printing of the analytical report according to file parameters. Reports are sent to the data cartridge if its output device number is entered into the file. In most instances, the analyst will want a copy of the report attached to the chromatogram so that both pieces of chromatographic information about the sample may be examined together. The report on data cartridge then becomes a duplicate and an additional task for the SP 4000 to accomplish before beginning the next run. In theory, obtaining a duplicate report requires only the change in output device, which is reset to the initial value after the duplicate report is completed. In practice, the process is somewhat more involved. For completely unattended operation, this change requires using a procedure. Because of the algorithm used by the SP 4000 to process data and print reports, each set of data must be processed according to the file before each report is generated.[2]

The procedural sequence used to obtain duplicate reports is built around the basic analytical sequence described above. A flow chart for this type of procedure is given in Figure 3. Sample data are acquired, saved in procedure storage, processed, and reported to the printer-plotter. The output device is reset to that of the data cartridge. The data from procedure storage are then processed through the file a second time and the duplicate report is recorded on the data cartridge. The entire sequence is repeated for each sample to be analyzed. The procedure is also used to initial all file parameters at the beginning of the

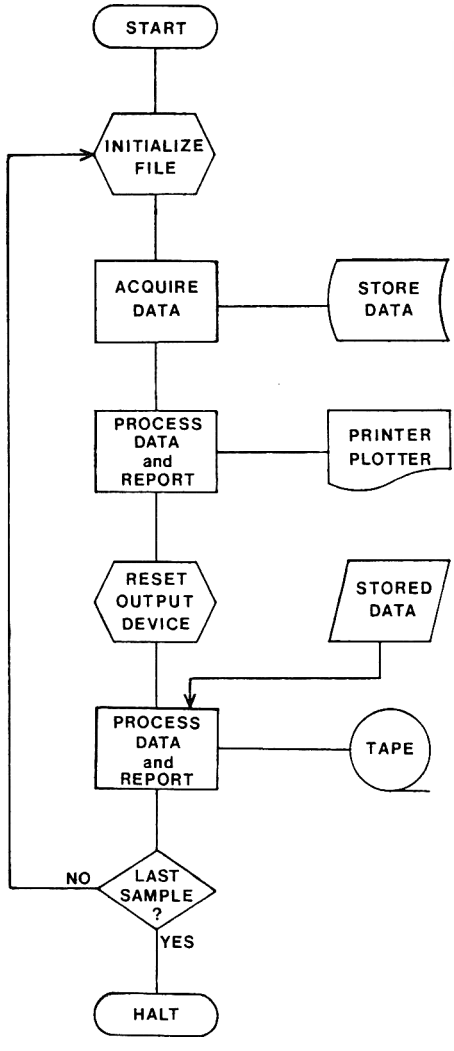


Figure 3. Flow chart of procedure for transfer to digital cartridge of duplicate reports for series of samples.

[2] The SP 4000 provides a command (RE) for re-calculation of results, which prints a duplicate report. This command was unsuitable for use with statistical reports. Since this type of report is often used by analysts using the system, it was excluded from all use in the procedures developed for report transfers.

```
MULT INJ TAPE TEST          1980 DEC 22 10:00:19

CHANNEL   5    RUN   3    FILE   5    METHOD  5

INDEX   3    SAMPLE  1-1 STD NUMBER 1

NAME          AVERAGE     REL SD %     STD DEV      VARIANCE

JUNK
HCB
HEPTACLOR  73370             .335      245.5      60070
CPFS
HEP EP
DIELDRIN
ENDRIN
P,P'-DDT
```

Figure 4. Typical statistical report prepared by using data from abbreviated report.

sequence. Having this chore done automatically under procedure control not only saves time but eliminates a potential source of operator error. Once this procedure has been entered into the SP 4000, only one change is usually required for its use in any subsequent run. The number of samples to be analyzed in the run must be respecified if it is different from the number of samples analyzed when the procedure was last used. A sample procedure based on this flow chart is reproduced and discussed in more detail in Appendix A.

The SP 4000 can be programmed to correlate multiple sets of injection data from replicate injections of a single sample into a single "abbreviated" report. In a typical abbreviated report, some information documenting system parameters is omitted. In addition, only concentration data from peaks identified by retention window names are given. After each injection, the new set of integration data is processed by the file and included in the report. The index number given in the report corresponds to the injection sequence number of the current set of data. Concentration data such as these may be further processed to produce a "statistical" report such as that given in Figure 4. An abbreviated report may be obtained without the corresponding statistical report if desired. The combined report provides a secondary advantage of an automated laboratory data system. Information relating to the precision of the determinative step of an analytical method, as well as the individual quantitation, may be obtained with no additional work by the analyst.

A combined abbreviated-statistical report, or any other type, may be further consolidated without loss of critical information by using a procedure to control data acquisition. A procedure's ACQUIRE step may be set up to inhibit all but the last report for a sequence of multiple injections of a sample. Three chromatograms, for example, followed by a single abbreviated and statistical report avoid repetitious reports and conserve the relatively expensive thermal paper used by the printer-plotter.

Transfer of an abbreviated-statistical report to the data cartridge requires that each set of data be stored, then sequentially processed by the file for a report to any output device. Intermediate reports are output to the cathode ray tube (CRT) of the CPU to allow proper termination of each chromatographic run.[3] A flow chart showing the required procedural sequence for triplicate injection reports is given in Figure 5. After initialization of file parameters, the next sample number is checked. When all samples have been analyzed, the procedure halts. In all other cases the system accepts the next injection as the first of 3 injections of the sample.

The sample number is stored for use in the final report. After each injection, integration data are stored in a selected procedure storage location and processed by the file, and a number of reports are output to the CRT. The file output device is then set for the printer-plotter and the first 2 sets of data are retrieved and processed. No reports are generated. The third set of data is then retrieved and processed, and a report is output to the printer-plotter. Following a check of the file output device to determine the destination of the last report, the report sequence is repeated for the data cartridge, or the entire procedure sequence is repeated for the next sample. A typical procedure based on this flow

[3] Appendix B contains a more detailed description of the need for reports output to the CRT.

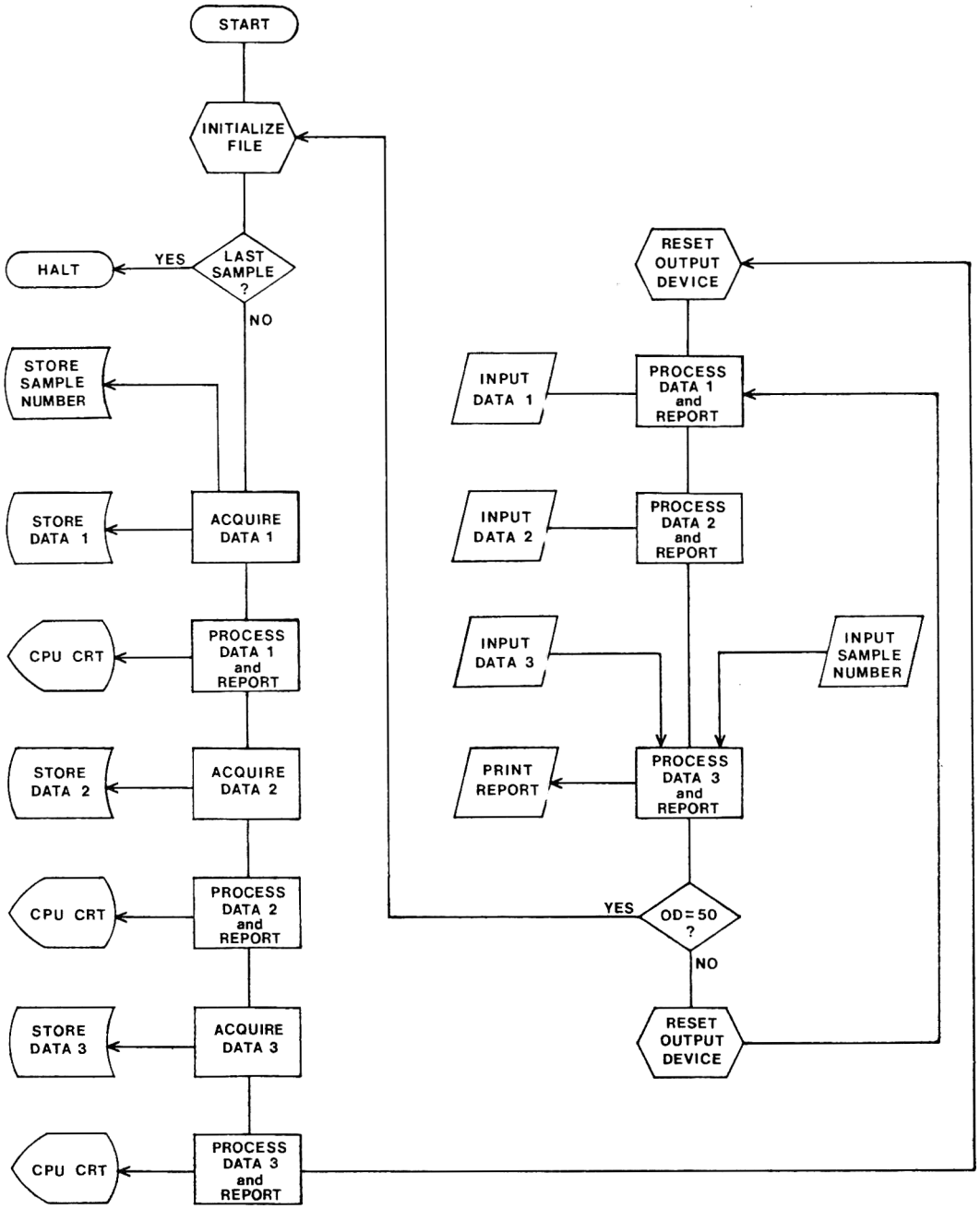


Figure 5. Flow chart of procedure to transfer abbreviated-statistical reports of triplicate analyses of sample to any 2 output devices.

chart is given and discussed in greater detail in Appendix B.

Recovery and Use of Taped Reports

Reports recorded on a data cartridge can serve as a compact duplicate record of chromatographic analyses. Reading of the reports on the data cartridge usually requires a second digital data cartridge unit, as well as a computer terminal. However, the data cartridge unit interfaced to the SP 4000 may be used, provided that it is compatible with the terminal. In either case, no

```
FISH EXTRACT COMPARISON      1981 JUL 13 22:05:48

CHANNEL   5     RUN   7     FILE   5     METHOD  5

VIAL   7      TRAY  2

INDEX  14     SAMPLE  7-2 15 169-1 NFF *B

S.AMT=

NAME            CONC        RT      AREA          KF

   1                         69      1878
   2                        269      1017
PARATHION     15470         296     15468          1
DIELDRIN     134800         549    134804          1
ENDRIN        27480         613     27477          1
   6                         892      4395
MOXYCHLOR      1758        1344      1758          1

TOTALS       179500
```

Figure 6. Typical report as it appears in ASCII characters on printer-plotter or transferred from data cartridge to computer terminal.

computer is required; reports are transferred from the data cartridge to the terminal in the local mode. For all practical purposes, the appearance of the report at the terminal will be virtually identical to the report printed at the printer-plotter. Some minor characters may be different if the terminal character set does not precisely match the upper case ASCII character set used by the SP 4000. Terminals with an automatic line feed feature will add an extra blank line after each line of the report unless this feature is turned off. A typical report as it appears at the printer-plotter and after transfer from the data cartridge to a terminal is shown in Figure 6.

More important, the data cartridge provides a convenient medium for error-free transfer of reports to a more powerful computer than the SP 4000. Once entered into that computer, unlimited additional processing and data reduction become available. The data cartridge reports are entered into the computer in much the same manner as used for reading at a terminal. The data cartridge unit and terminal must be on-line with the computer. A computer program entering the reports into the computer is executed before the data cartridge is read.

In this application the computer programming language APL (*A Programming Language*, 6–8) running on one of 2 IBM 370/168 Model 3 central processor units at the Parklawn Computer Center is used to process the reports. APL is a conversational, time-sharing language capable of processing numeric or alphabetic data. It has a large set of primitive operators and a powerful array of processing capability to simplify matrix operations. An unusual syntax and character set, as well as understandable error messages, make program construction and use a relatively simple task. A large variety of graphics and statistical software packages are also available.

The SP 4000 reports are composed entirely of character data—letters, digits, and punctuation. Before data contained in the reports can be processed by the computer they must be translated into APL language. This translation[4] is carried out by the computer as the reports are read from the data cartridge. Each ASCII character is replaced by its corresponding APL character. Figure 7 shows the SP 4000 report as it appears in APL characters before translation into the computer language. The same report after translation, except for the italicized alphabet, appears identical to the original ASCII version shown in Figure 6. All of the information contained in the reports is now available to the computer. Character digits can be converted to numeric data and used in calculations. Chemical and sample names can be stored for correlation with integration data produced by the SP 4000. In addition, the translated reports, as well as all reduced data, can be saved in computer memory for future reference or recorded on magnetic tape at the computer center or a data cartridge in a

[4] For a detailed discussion of the ASCII to APL translation and similar processing of data from an Inductively Coupled Plasma, see Capar, S.G., & Dusold, L.R. (1978) *Am. Lab.* **10**, 17–27 passim.

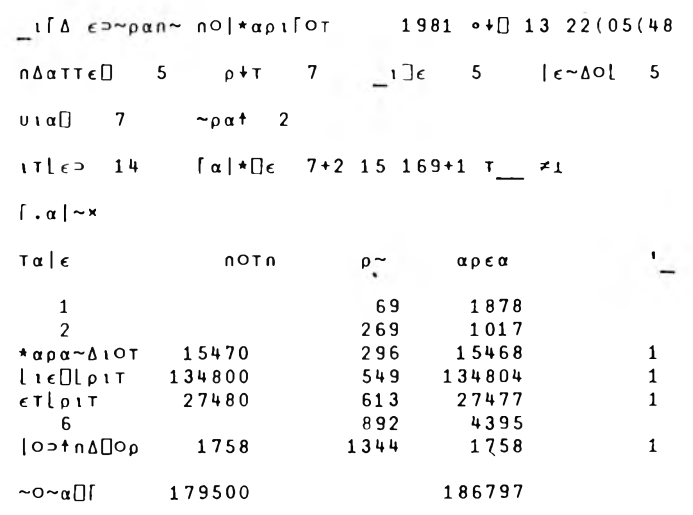


Figure 7. Typical report as it appears in APL characters before translation into APL.

laboratory for archival storage. Computer programs have been written in APL to process integration data for both the single and the abbreviated-statistical report formats. Specifically, the programs are designed to produce detailed reports of sample analyses for pesticide residues from chromatography of solutions containing sample extracts in series with solutions containing the corresponding reference standards.

Sample information is entered into a SP 4000 file which contains proper parameters for integration of peaks detected in the type of chromatography to be used for the analysis. Autosampler tray position and a quantitation code are coded into the sample name. The quantitation code consists of an asterisk followed by a specific letter. All injections of a given sample solution and the standard solution to be used for quantitation of residues found in that sample are given the same quantitation code. The code is used by the programs to correlate injections of 2 or more solutions. After the run is completed, the reports are transferred from the data cartridge to the computer, translated into APL, and stored. Autosampler tray position for each vial is compared with that given in the sample name. Reports containing codes that do not match are brought to the attention of the analyst who may keep or discard the data. All pertinent information available in the reports is extracted, grouped according to type, and stored. Separate lists of sample names, pesticide names, and detector response to sample and standard solutions are compiled for use in the programs, but are also readily accessible for other purposes.

Several types of information required for residue quantitation cannot be entered into the SP 4000. This information is readily entered into the computer through the interactive nature of the APL language. Pesticide concentrations in standard solutions, weights of pesticides used for fortification of samples, and some sample data (e.g., analysis weight and final volume) are entered in response to prompting by the computer. Redundant checks of the accuracy of information entered by the analyst have been included throughout the programs to avoid errors that otherwise may go unnoticed. The analyst is asked if the samples were fortified before fortification data are requested. After entry, the data are displayed so that a check of the accuracy may be made. Any errors detected are corrected before proceeding to the next operation.

The data necessary for quantitative calculations of the concentrations of all residues detected in all samples are now stored in the computer. All calculations are completed in a matter of several seconds. A title for all analyses is entered, and the comprehensive analytical reports generated by the computer are printed at the terminal. Figure 8 contains a typical computer-generated report of sample analysis. In this example, a fish sample was analyzed for fortified and biologically incorporated pesticide residues by the method for nonfatty foods in the *Pesticide Analytical Manual* (6). The report contains all numeric data rounded to 3 significant figures. The analyst-entered sample information is followed by 2 sections containing sample and standard data. The analyst must determine

FISH EXTRACTION COMPARISON----WHITE PERCH NON-FATTY FOOD METHOD
PAM 212 ANALYSIS OF 15 169-1 NFF *B

****

20 JULY 1981 15°/° ELUATE

SAMPLE WEIGHT: 22.8 CHROMATOGRAPHED: 13 JUL 1981
ML CH3CN: ADDED--217, REC'D--205
ML PET ETHER REC'D: 88.5 G ONTO FLORISIL: 19.1
ML FINAL VOL: 5.0 MG SAMPLE EQUIV: 19.1

SAMPLE SOLUTION DATA

	PARATHION	DIELDRIN	ENDRIN	MOXYCHLOR
°/° RECOVERY	102	-	109	-
PPM FOUND	.259	.52	.104	.0164
NG FOUND	4.93	9.91	1.98	.313
MEAN RES	15500	135000	27500	1730
SD (MINIMUM)	15500	135000	27500	1700
CV (MAXIMUM)	15500	135000	27500	1760
UG ADDED	5.77	0	2.17	0
PPM ADDED	.253	0	.0954	0

STANDARD SOLUTION DATA

	PARATHION	DIELDRIN	ENDRIN	MOXYCHLOR	MIREX
NG INJECTED	4.91	9.75	1.85	8.35	7.4
UG/ML	.982	1.95	.37	1.67	1.48
STD NFF *B----------			5 UL INJECTIONS		
MEAN RES	15400	133000	25700	46100	75200
MIN RES	15300	132000	25400	45500	75000
MAX RES	15400	133000	26000	46700	75400

Figure 8. Comprehensive APL report of analysis generated from data in taped SP 4000 reports supplemented by additional information entered into computer by analyst.

if response data for peaks detected in the sample solution correspond to maximum and minimum or standard deviation (SD) and coefficient of variation (CV) values. This determination can normally be made by comparison of the orders of magnitude of these data with the mean response. In this report, equal orders of magnitude imply that 2 injections of the sample solution were made. All other information does not require interpretation beyond that normally required for this type of analysis. A report similar in format to that given in Figure 8 is printed for samples analyzed by any other method. In this more general case, the only sample information required is the analytical sample weight and corresponding final volume.

In many series of analyses the analyst must compare selected data from each of the reports. Extraction and tabulation from comprehensive computer-generated reports is a time-consuming task for the analyst. A supplemental computer program has therefore been written to carry out this chore. A typical computer summary report of percent recovery is given in Figure 9. Any of the 8 items listed in the sample solution data section of a report of analysis (Figure 8) may be tabulated in a summary report of this type.

The entire computer-assisted laboratory data

FISH EXTRACTION COMPARISON----WHITE PERCH
PAM 212 ANALYSES SUMMARY OF: °/° RECOVERY
14 JULY 1981 CHROMATOGRAPHED: 13 JUL 1981

	PARATHION	DIELDRIN	ENDRIN	MOXYCHLOR
6 169-1 NFF *A	-	-	-	101
15 169-1 NFF *B	94.1	-	100	-
6 169-2 NFF *C	-	-	-	98.4
15 169-2 NFF *D	91.6	-	101	-

	MIREX
6 169-1 NFF *A	88.3
15 169-1 NFF *B	-
6 169-2 NFF *C	84.6
15 169-2 NFF *D	-

Figure 9. Typical computer-generated summary of data selected from series of analyses.

processing system has been used with little difficulty by a number of analysts in this laboratory. The minimal knowledge of computer programs and operations required for utilization of this system makes it ideal for laboratory data management.

Acknowledgments

The able assistance of L. R. Dusold and S. G. Capar, Food and Drug Administration, in the implementation of the SP 4000-Tektronix 4923-01 interface and APL programming is gratefully acknowledged.

References

(1) *SP 4000 Chromatography Data System Instruction Manual* (Rev. 3/77), Part No. A0098-01, Spectra-Physics, 2905 Stender Way, Santa Clara, CA 95051

(2) Tektronix, Inc., PO Box 500, Beaverton, OR 97077

(3) *APL Language*, Publication GC26-3847-4, IBM Corp., PO Box 50020, San Jose, CA 95150

(4) *APL Shared Variables*, Publication SH20-9087-0. IBM Corp., PO Box 50020, San Jose, CA 95150

(5) Gilman, L., & Rose, A. J. (1976) *APL: An Interactive Approach*, 2nd Ed., John Wiley and Sons, New York, NY

(6) *Pesticide Analytical Manual*, Vol. I, U.S. Food and Drug Administration, Washington, DC, section 212

APPENDIX A

Procedure for Transfer of Single Injection Reports to Magnetic Tape

This procedure has been used to transfer a duplicate report to magnetic tape. File 5, output device 5, and channel 5 are original SP 4000 components. The tape unit is output device 50. All commands are echoed at the CPU's CRT (output device 54) because of the slow print rate of the printer-plotter with respect to CPU operations. Several WAIT steps in the procedure ensure that each device is not busy before it is used. Several COMMAND steps are used to execute 2 commands each by entering the step with a blank between the 2 commands. The number 13 (hexadecimal equivalent for "enter") is then entered into the work register corresponding to the blank space. When printed at any output device other than the CRT, the second command will appear on a separate line from the first command in the step (e.g., Step 3).

Step 1 is extra and has been used as a COMMAND step to execute a procedure listing chromatographic conditions before beginning a run. Steps 2–6 set up the file for the first sample analysis. In step 7, data are acquired and stored in procedure storage location 1 (W2), and a report is printed at output device 5 (the printer-plotter) as part of the file's normal operation. Steps 8 and 9 reset the output device for the report to the tape made in Step 11. Step 13 enters the file in preparation for changing the output device. Step 14 jumps directly to Step 5 (W5), incrementing the jump counter (W2) in the process. Since the file was entered before the jump, Step 5 can change the output device for the next injection. This sequence is continued until the value of the jump counter (W2) of Step 14 is the same as the desired number of jumps (samples). At this point, no jump is made. The file output device is reset to its original value (5) in Step 15,

```
PROCEDURE 005      STEP 001
ACQUIRE     DELAY 0.000
F0 0    F1 0    F2 0    F3 0
F4 0    F5 0    F6 0    F7 0
W0 000    W1 000    W2 000
W3 000    W4 000    W5 000
W6 000    W7 000    W8 000

PROCEDURE 005      STEP 002
COMMAND     AT5
F0 0    F1 0    F2 1    F3 0
F4 1    F5 0    F6 1    F7 0
W0 006    W1 054    W2 065
W3 084    W4 053    W5 032
W6 032    W7 032    W8 032

PROCEDURE 005      STEP 003
COMMAND     FI5
SN1
F0 1    F1 0    F2 0    F3 1
F4 0    F5 0    F6 1    F7 0
W0 006    W1 054    W2 070
W3 073    W4 053    W5 013
W6 083    W7 078    W8 049

PROCEDURE 005      STEP 004
COMMAND     RN0
IX1
F0 0    F1 1    F2 1    F3 1
F4 0    F5 0    F6 1    F7 0
W0 006    W1 054    W2 082
W3 078    W4 048    W5 013
W6 073    W7 088    W8 049

PROCEDURE 005      STEP 005
COMMAND     OD5
FF
F0 0    F1 0    F2 1    F3 0
F4 0    F5 0    F6 1    F7 0
W0 006    W1 054    W2 079
W3 068    W4 053    W5 013
W6 070    W7 070    W8 032

PROCEDURE 005      STEP 006
COMMAND     XD1
XX
F0 0    F1 0    F2 1    F3 0
F4 0    F5 0    F6 1    F7 0
W0 006    W1 054    W2 088
W3 068    W4 049    W5 013
W6 088    W7 088    W8 032

PROCEDURE 005      STEP 007
ACQUIRE     DELAY 0.000
F0 0    F1 0    F2 1    F3 0
F4 0    F5 0    F6 0    F7 0
W0 000    W1 005    W2 001
W3 004    W4 000    W5 000
W6 000    W7 000    W8 000

PROCEDURE 005      STEP 008
COMMAND     FI5
F0 1    F1 0    F2 0    F3 1
F4 0    F5 0    F6 1    F7 0
W0 006    W1 054    W2 070
W3 073    W4 053    W5 032
W6 032    W7 032    W8 032

PROCEDURE 005      STEP 009
COMMAND     OD50
XX
F0 0    F1 0    F2 1    F3 0
F4 0    F5 0    F6 1    F7 0
W0 006    W1 054    W2 079
W3 068    W4 053    W5 048
W6 013    W7 088    W8 088

PROCEDURE 005      STEP 010
WAIT        DELAY 0.000
F0 0    F1 0    F2 0    F3 0
F4 0    F5 0    F6 0    F7 0
W0 002    W1 000    W2 005
W3 000    W4 000    W5 000
W6 000    W7 000    W8 000

PROCEDURE 005      STEP 011
REPORT
F0 0    F1 0    F2 1    F3 0
F4 0    F5 0    F6 0    F7 0
W0 001    W1 005    W2 001
W3 004    W4 000    W5 001
W6 000    W7 000    W8 000

PROCEDURE 005      STEP 012
WAIT        DELAY 0.000
F0 0    F1 0    F2 0    F3 0
F4 0    F5 0    F6 0    F7 0
W0 002    W1 000    W2 005
W3 000    W4 000    W5 000
W6 000    W7 000    W8 000

PROCEDURE 005      STEP 013
COMMAND     FI5
F0 1    F1 0    F2 0    F3 1
F4 0    F5 0    F6 1    F7 0
W0 006    W1 054    W2 070
W3 073    W4 053    W5 032
W6 032    W7 032    W8 032

PROCEDURE 005      STEP 014
JUMP
F0 0    F1 0    F2 0    F3 0
F4 0    F5 0    F6 0    F7 0
W0 003    W1 000    W2 000
W3 000    W4 011    W5 005
W6 000    W7 000    W8 000

PROCEDURE 005      STEP 015
COMMAND     OD5
XX
F0 0    F1 0    F2 1    F3 0
F4 0    F5 0    F6 1    F7 0
W0 006    W1 054    W2 079
W3 068    W4 053    W5 013
W6 088    W7 088    W8 032

PROCEDURE 005      STEP 016
MESSAGE     STOP
F0 0    F1 0    F2 1    F3 0
F4 1    F5 0    F6 1    F7 0
W0 005    W1 050    W2 083
W3 084    W4 079    W5 080
W6 032    W7 032    W8 032
```

since most runs not under procedure control require a report at the printer-plotter. The message "STOP" that is used by the computer programs is recorded on the tape (W1 = 50) in the final step of the procedure.

APPENDIX B

Procedure for Transfer of Duplicate Abbreviated and Statistical Reports to Magnetic Tape

This procedure has been used to transfer duplicate combined abbreviated and statistical reports to magnetic tape. The same data system components and general comments given in Appendix A apply here.

Steps 1 through 4 initial the file. Only a few file parameters must be set initially, because many are forced to the desired values by steps used to accomplish other tasks later in the procedure. Each run is begun by the autosampler signal to the "run set" screw terminal at the DIM. The run is ended by using the T6 time function to signal the "run re-set" screw terminal. In the time file, T6 is set to turn on to end the run, then turn off 1 s later during the report. If T6 is not turned off, each new run begun by the autosampler signal is immediately ended by T6, which is still on. All samples will be injected by the autosampler but no chromatograms or data will be processed. This is exactly what occurs when no report is made by the file for an individual injection made under control of an ACQUIRE step as used in this procedure. The T6 time function will not turn off unless a report is being made at the same time. This problem is easily avoided by output of a relatively large number (20) of reports to the CRT message line (output device 54). In spite of the speed of the CPU, this operation requires several seconds and allows enough time for T6 to be turned off during a report.

Step 6 is used as a sample counter. A jump to Step 8 is made until the counter (W2) equals the W4 value, the number of samples plus 1. At this time, the procedure passes through the JUMP and stops at Step 7. Otherwise, Step 8 stores the sample number (W2 of Step 6) in Step 18. Steps 9, 10, and 11 each acquire data from a single injection and store it in the procedure storage location specified in W2 of each step. Steps 12 and 13 reset the file output device for the report to the printer-plotter. An abbreviated report with statistics requires that each set of data be processed through the file individually. Steps 14

```
PROCEDURE 003      STEP 001
COMMAND     AT5
F0 0    F1 0    F2 1    F3 0
F4 1    F5 0    F6 1    F7 0
W0 006      W1 054      W2 065
W3 084      W4 053      W5 032
W6 032      W7 032      W8 032

PROCEDURE 003      STEP 002
COMMAND     FI5
RNO
F0 1    F1 0    F2 0    F3 1
F4 0    F5 0    F6 1    F7 0
W0 006      W1 054      W2 070
W3 073      W4 053      W5 013
W6 082      W7 078      W8 048

PROCEDURE 003      STEP 003
COMMAND     OD54
F0 0    F1 0    F2 1    F3 0
F4 0    F5 0    F6 1    F7 0
W0 006      W1 054      W2 079
W3 068      W4 053      W5 052
W6 032      W7 032      W8 032

PROCEDURE 003      STEP 004
COMMAND     NR20
XX
F0 0    F1 1    F2 0    F3 0
F4 1    F5 0    F6 1    F7 0
W0 006      W1 054      W2 078
W3 082      W4 050      W5 048
W6 073      W7 088      W8 088

PROCEDURE 003      STEP 005
WAIT        DELAY 5.000
F0 0    F1 0    F2 0    F3 0
F4 0    F5 0    F6 0    F7 0
W0 002      W1 000      W2 000
W3 000      W4 000      W5 000
W6 000      W7 005      W8 000

PROCEDURE 003      STEP 006
JUMP
F0 0    F1 0    F2 0    F3 0
F4 0    F5 0    F6 0    F7 0
W0 003      W1 000      W2 000
W3 000      W4 011      W5 008
W6 000      W7 000      W8 000

PROCEDURE 003      STEP 007
HALT
F0 1    F1 0    F2 0    F3 0
F4 0    F5 0    F6 0    F7 0
W0 004      W1 000      W2 000
W3 001      W4 000      W5 000
W6 000      W7 000      W8 000

PROCEDURE 003      STEP 008
NUMBER
F0 0    F1 1    F2 0    F3 0
F4 0    F5 0    F6 0    F7 0
W0 007      W1 005      W2 000
W3 002      W4 000      W5 018
W6 154      W7 000      W8 000

PROCEDURE 003      STEP 009
ACQUIRE     DELAY 0.000
F0 0    F1 0    F2 1    F3 0
F4 1    F5 0    F6 0    F7 0
W0 000      W1 005      W2 001
W3 020      W4 000      W5 000
W6 000      W7 000      W8 000

PROCEDURE 003      STEP 010
ACQUIRE     DELAY 0.000
F0 0    F1 0    F2 1    F3 0
F4 0    F5 1    F6 0    F7 0
W0 000      W1 005      W2 002
W3 036      W4 000      W5 000
W6 000      W7 000      W8 000

PROCEDURE 003      STEP 011
ACQUIRE     DELAY 0.000
F0 0    F1 0    F2 1    F3 0
F4 1    F5 1    F6 0    F7 0
W0 000      W1 005      W2 003
W3 052      W4 000      W5 000
W6 000      W7 000      W8 000

PROCEDURE 003      STEP 012
COMMAND     FI5
OD5
F0 1    F1 0    F2 0    F3 1
F4 0    F5 0    F6 1    F7 0
W0 036      W1 054      W2 070
W3 073      W4 053      W5 013
W6 079      W7 068      W8 053

PROCEDURE 003      STEP 013
COMMAND     XX
F0 0    F1 0    F2 0    F3 1
F4 1    F5 0    F6 1    F7 0
W0 006      W1 054      W2 088
W3 088      W4 032      W5 032
W6 032      W7 032      W8 032

PROCEDURE 003      STEP 014
REPORT
F0 0    F1 0    F2 1    F3 0
F4 1    F5 0    F6 0    F7 0
W0 001      W1 005      W2 000
W3 020      W4 000      W5 001
W6 000      W7 000      W8 000

PROCEDURE 003      STEP 015
REPORT
F0 0    F1 0    F2 1    F3 0
F4 0    F5 1    F6 0    F7 0
W0 001      W1 005      W2 000
W3 036      W4 000      W5 002
W6 000      W7 000      W8 000

PROCEDURE 003      STEP 016
COMMAND     FI5
F0 1    F1 0    F2 0    F3 1
F4 0    F5 0    F6 1    F7 0
W0 006      W1 054      W2 070
W3 073      W4 053      W5 032
W6 032      W7 032      W8 032

PROCEDURE 003      STEP 017
COMMAND     SN
F0 0    F1 1    F2 1    F3 1
F4 0    F5 0    F6 1    F7 0
W0 006      W1 054      W2 083
W3 078      W4 032      W5 032
W6 032      W7 032      W8 032

PROCEDURE 003      STEP 018
COMMAND     001
F0 0    F1 0    F2 0    F3 0
F4 1    F5 1    F6 0    F7 0
W0 006      W1 200      W2 048
W3 048      W4 049      W5 032
W6 032      W7 032      W8 032

PROCEDURE 003      STEP 019
COMMAND     XX
F0 0    F1 0    F2 0    F3 1
F4 1    F5 0    F6 1    F7 0
W0 006      W1 054      W2 088
W3 088      W4 032      W5 032
W6 032      W7 032      W8 032

PROCEDURE 003      STEP 020
REPORT
F0 0    F1 0    F2 1    F3 0
F4 1    F5 1    F6 0    F7 0
W0 001      W1 005      W2 001
W3 052      W4 000      W5 003
W6 000      W7 000      W8 000

PROCEDURE 003      STEP 021
JUMP
F0 1    F1 0    F2 0    F3 0
F4 0    F5 0    F6 0    F7 1
W0 003      W1 005      W2 000
W3 129      W4 000      W5 002
W6 009      W7 000      W8 050

PROCEDURE 003      STEP 022
COMMAND     FI5
F0 1    F1 0    F2 0    F3 1
F4 0    F5 0    F6 1    F7 0
W0 006      W1 054      W2 070
W3 073      W4 053      W5 032
W6 032      W7 032      W8 032

PROCEDURE 003      STEP 023
COMMAND     OD50
XX
F0 0    F1 0    F2 1    F3 0
F4 0    F5 0    F6 1    F7 0
W0 006      W1 054      W2 079
W3 068      W4 053      W5 048
W6 013      W7 088      W8 088

PROCEDURE 003      STEP 024
JUMP
F0 0    F1 0    F2 0    F3 0
F4 0    F5 0    F6 0    F7 0
W0 003      W1 000      W2 000
W3 000      W4 000      W5 014
W6 000      W7 000      W8 000
```

and 15 retrieve data from procedure storage (W5) and process it while forcing file indexing parameters to the proper values with flag register entries. In both cases no report is made (W2 = 0). The proper sample number is then entered into the file by Steps 16–19.

The final report is prepared in Step 20. The third set of data is retrieved from storage location 3 (W5) and processed with appropriate file parameters, and 1 copy (W2) is output to the device specified in the file (the printer-plotter).

JUMP Step 21 makes a logical comparison of the value in W8 and the current file output device number. Since the file value is less than the W8 value, a report to the tape has not been made. The file is then entered, the output device is reset to 50, and a direct jump to the beginning of the report sequence (Step 14) is made in Steps 22–24. When the file value (50) is greater than or equal to the W8 value (50), a jump to Step 2 is made. Reports to both the tape (device 50) and the printer-plotter (device 5) have been made and the cycle begins for the next sample.

Development of a Chemical Information System

G. W. A. MILNE
National Cancer Institute, Information Technology Branch, Bethesda, MD 20205

The 10-year history of the development of the NIH-EPA Chemical Information System is described. The System grew from the cooperative efforts of several agencies of the federal government and now is publicly available on a fee-for-service basis on computers in the private sector.

Mass Spectral Search System (MSSS)

By 1969, the Laboratory of Chemistry of the National Heart and Lung Institute of the National Institutes of Health (NIH) had become very heavily involved in the application of mass spectrometry to problems in biochemistry and medicine. A major capability of mass spectrometry in this and other areas is its ability to identify chemical substances from the mass spectra they produce. As more and more reference spectra became available, it became increasingly clear that the establishment of a mass spectral library together with computer programs that can search it efficiently could be very useful as an aid in the identification of unknown chemicals.

Accordingly, in a collaborative effort with the NIH Division of Computer Research and Technology (DCRT), a pilot "Mass Spectral Search System" (MSSS) was developed and made available for use by NIH scientists (1). This pilot system permitted the identification of compounds from their mass spectra. An example of such a procedure is shown in Figure 1. Here the user asks first to retrieve all spectra with a peak at m/z 207 having an intensity in the range between 100% and 100%, i.e., the intensity must equal 100%; this must be the "base peak" of the spectrum. In response, the system identifies 59 library spectra that meet this condition and they are stored in a temporary file No. 1. Searching with a second peak, m/z 209, intensity between 95% and 100%, reduces this number to 3. The third peak entered, m/z 224 with intensity between 70% and 100%, leads to the single compound in the library, 2,3,6-trichlorobenzoic acid, whose spectrum meets all 3 of these conditions. This reference spectrum can be retrieved in bar graph form, as shown in Figure 2, which can be used for direct comparisons of the unknown and reference spectra.

In addition to this interactive search capability, MSSS has a batch search program and also a reverse search feature. The former is based on algorithms of Hertz et al. (2) and the latter on programs from McLafferty's group (3). Both batch searches can be run on-line ("while you wait") or in an overnight procedure. In addition to the basic search programs, MSSS has a wide variety of other search capabilities, ranging from a proton affinity search system to programs which can calculate formulas from accurate masses.

The programs in MSSS are all conversational and the computation necessary for the various search and display functions was accomplished in real time on a DEC System 10 computer at DCRT.

During the decade since this project was initiated, the search system has been used by several hundred analytical chemists in many countries around the world. The applications of the system are numerous and include emergency toxicology, quality control, pollutant monitoring, and studies in biological disposition of chemicals.

Structure and Nomenclature Searching

As use of the Mass Spectral Search System increased, it soon became clear that although identification of a compound from its mass spectrum, as shown in Figures 1 and 2, was a useful function, the reverse task, identification and retrieval of the mass spectrum from a given structure, was also important. It was therefore decided to provide this capability as an adjunct to MSSS.

Richard Feldmann had been working at NIH since 1972 on the design of computer programs for retrieval of chemical structures (4) and the Chemical Information System (CIS) sought to capitalize on this achievement by the adoption of many of the techniques that had emerged from his work. Any chemical structure can be expressed in a purely alphanumeric form by using one of a variety of conventions (5). Computer programs have been written (6) for the interconversion of conventional structure diagrams and many of the alphanumeric equivalents.

Presented at the Symposium on Computers in the Laboratory, 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

0004-5756/82/6505-1249-10$01.00

```
Option? PEAK

Type peak, min int, max int
CR to exit, 1 for CAS RN, OI, MW, MF and Name

User: 207,100,100

File 1 contains 59 references to m/z 207

Next request: 209,95,100

File 2 contains 3 references to m/z 207 209

Next request: 224,70,100

File 3 contains 1 reference to m/z 207 209 224

Next request: 1

CAS RN          OI   MW  Formula, Names
  50-31-7      507  224  C7H3C1302
                         Benzoic acid, 2,3,6-trichloro- (8CI9CI)
                         Benzabar
                         Benzac
                         Benzac 1-281
                         Benzac 1,281
```

Figure 1. Interactive search through mass spectral data base.

Typically, such a record contains, for each atom in the structure, a string of digital information which identifies the atom type, its neighbors, and the types of bonds between it and its neighbors. A collection of such data for every non-hydrogen atom in the molecule is called a connection table and is equivalent to the structure of the molecule, a subset of the connection table of course being equivalent to a substructure. It follows that connection tables can be searched on a line-by-line basis and that herein lies a method of structure or substructure searching. This fact was exploited by the Feldmann programs, which were successfully merged into CIS in 1976 (7). This original version of the Structure and Nomenclature Search System (SANSS) subsequently underwent various modifications (8) as it evolved into the system which is currently in general use.

Every chemical in CIS is identified in terms of its Chemical Abstracts Service (CAS) Registry number. This task has been carried out by CAS under contract to the government and is done by searching the files of the CAS Registry using the name or the structure of the chemical. Once the correct entry is located, the Registry number is retrieved and is used in turn to retrieve the connection table and the nomenclature information. All these data are used by CIS to build the files which support the Structure and Nomenclature Search System. This effort, in addition to making possible the use of the CAS Registry number and structure for all chemicals in CIS, also serves as a check on the quality and accuracy of the nomenclature used in the files. As such, it has proved to be a worthwhile task, as it has allowed the resolution of numerous errors in the data base (9).

It is possible, by using these programs, to search the data base for any full structure or any partial structure, defined as one or more atoms and their neighbors and bonds. The structure or substructure being sought must be entered into the computer, as shown in Figure 3, and then specific search requests may be made. In Figure 3, an *m*-dichlorobenzene moiety is defined by the user with the help of simple commands such as RING, ALTBD, and so on, which the computer interprets and uses to build a con-

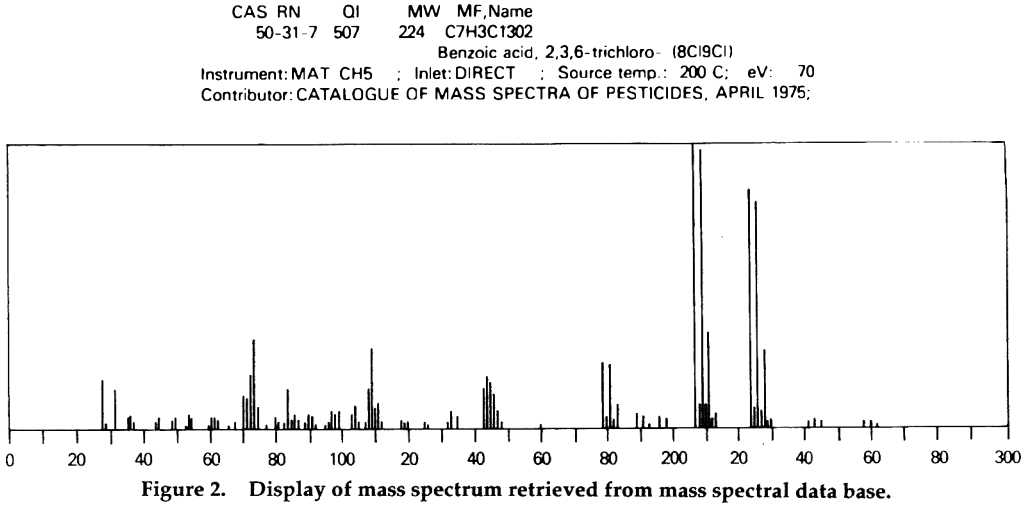
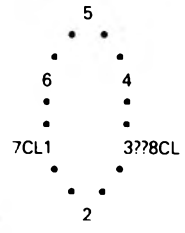


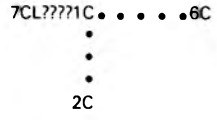
Figure 2. Display of mass spectrum retrieved from mass spectral data base.

Option? RING
Option? ALTBD 1 2
Option? ABRAN 1 AT 1 1 AT 3
Option? SATOM 7 8
Specify element symbol = CL
Option? D



Option? FPROB 1 3

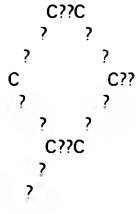
Fragment:



Required occurrences for hit: 2
This fragment occurs in 3258 compounds

File = 1, 3258 compounds contain this fragment

Option? RPROBE



	Conditions of search
Characteristics to be matched	Type of match
Type of ring or nucleus	EXACT
No heteroatoms	EXACT
Substituents	IMBED
This ring/nucleus occurs in 21844 compounds	

File = 2, 21844 compounds contain this ring/nucleus

Figure 3. Atom-centered fragment and ring search for *m*-dichlorophenyl moiety.

nection table. Once the "query structure" is complete, the user displays it as in Figure 3 and then asks (FPROBE 1 3) to retrieve all compounds which contain an atom identical to atom 1 and also an atom identical to atom 3. Stated differently, the system is being asked to find in the data base all compounds containing 2 or more chlorine atoms attached to aromatic rings. This is accomplished in a matter of seconds, and 3258 compounds are retrieved and stored in tempo-

Option? #1 AND #2

File: 3 Count: 2269

Option? SUBSS 3

Doing sub-structure search
Type E to Exit

File entry 20, Hits so far 13
File entry 40, Hits so far 28
File entry 60, Hits so far 39

File entry 2240, Hits so far 1346
File entry 2260, Hits so far 1356

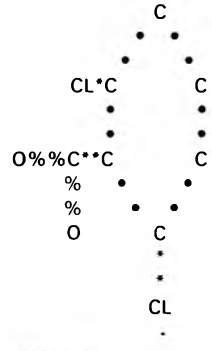
File = 4 Successful sub structures = 1361

Option? SSHOW 4

How many (E to Exit)? 2

Type E to terminate display.

Entry 1 CAS RN 50-30-6



Entry 2 CAS RN 50-31-7

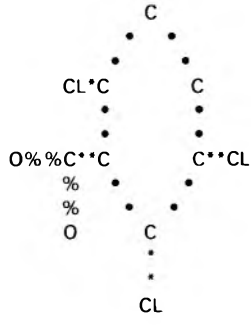


Figure 4. Substructure search for *m*-dichlorophenyl moiety.

Option? IDENT

File 5. This structure is contained in 1 compounds.

Option? SSHOW 5

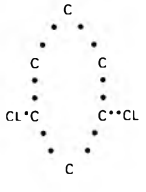
Entry 1 CAS RN 541-73-1
CIS Sources Of Information

2 - CIS, EI Mass Spectrometry
3 - CIS, Carbon-13 NMR Spectrometry
115 - EPA/CIS, WaterDROP

Non-CIS References

1 - EPA, TSCA Inventory List
7 - Merck Index: 3028
11 - EPA, AEROS SOTDAT: 5806
17 - NBS/NIH, Ionization Potential
19 - FDA/EPA, Pesticides Ref. Standards: 244
21 - U.S. International Trade Commission PEI Statistics: IMP/T03-77,PROD/T03
25 - EPA, Effluent Guidelines Priority Pollutants
26 - EPA, Organic Chemical Producers: 1215
30 - EROICA, Organic Properties
35 - ORNL, EMIC
53 - API/TRC, Thermodynamics and Spectroscopy
77 - NLM, CHEMLINE: TOXLINE,TDB,TOXBACK
112 - Aldrich Catalog/Handbook: 113808

C6H4C12



Benzene, 1,3,-dichloro- (9CI)
Benzene, m-dichloro- (8CI)
m-Dichlorobenzene
m-Dichlorobenzol
m-Phenylene dichloride
1,3-Dichlorobenzene

Figure 5. Full structure search for *m*-dichlorobenzene.

rary file No. 1. The only property common to these 3258 compounds is that they all must possess 2 chlorines attached to aromatic rings; the position of these atoms is not specified. In a second search (RPROBE), the user seeks all compounds that contain a 1,3-disubstituted aromatic ring. Extra substituents are allowed and the system finds 21 844 compounds meeting this condition. These are stored in file No. 2. When the contents of file No. 1 are combined with those of file No. 2, as shown in Figure 4, file No. 3 is created, containing 2269 compounds that each contain 2 chlorophenyl fragments and a 1,3-disubstituted ring. Some but not necessarily all of these compounds will contain a 1,3-dichloro aromatic system.

To eliminate those compounds that meet the search criteria but are not 1,3-dichloro aromatic compounds, a "substructure search" through this set of 2269 compounds must be carried out as in Figure 4. This leads to 1361 compounds in file No. 4 which are all in fact 1,3-dichloro aromatic compounds. The first 3 retrieved structures are shown in Figure 4.

CIS Chemical Locator Function (CLF)

The exact structure of a compound is a special case of a substructure and thus the addition of a substructural searching capability to the system met the immediate requirement in that compounds could be retrieved from the data base by structure. Substructure searching is, however, a relatively inefficient way to retrieve an exact match to a chemical structure and so a different approach to full structure searching was adopted. In this method, the full query structure is hash-encoded and the resulting code is passed against a copy of the structure file in which every entry has been similarly hash-encoded (10). This procedure finds and retrieves the correct structure reliably and rapidly with a single pass through the data base, as may be seen from the example in Figure 5, in which a full structure search for *m*-dichlorobenzene is carried out and leads only to that exact structure, whose record, beginning with the Registry number 541-73-1, is then printed at the terminal. The Chemical Locator Function of SANSS displays the files which carry data on the compound, and the remainder of the record contains the compound molecular and structural formulas and all its names, which include 2 systematic names, the CAS Ninth Collective Index (9CI) and Eighth Collective Index names, and 4 synonyms.

A second additional capability which was provided was that of nomenclature searching. Searching for chemicals by name has some well known deficiencies which stem from ambiguities in the rules for chemical nomenclature. Otherwise, it is a convenient means of locating complex chemicals rapidly. As an example, handling complex structures, such as those of natural products, on a computer is far more difficult and time-consuming than searching with trivial names such as "morphine" or "cholesterol." Consequently, name searching appears to complement structure searching nicely and this has been clearly evident in CIS, which, since 1978, has been the only public system in the world to offer both name searching and structure searching (11). A useful nomenclature search capability must be able to handle complete or partial names, e.g., "benzyl chloride" or "benzyl" with or without "chloride." Truncation of a word, either on the left ":enzyl," or the right "benz:" must also be possible.

All of these variations may be used in CIS, an example of whose name searching capability is

Option? NPROBE

Fragment or whole name search (F/W) (F)?W

Specify name (CR to exit): TCDD

File 1, 1 compounds having name: TCDD
Specify name (CR to exit):

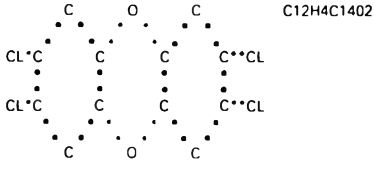
Option? SSHOW 1

Entry 1 CAS RN 1746-01-6
CIS Sources of Information

2 - CIS Mass Spectrometry
6 - Cambridge Xray Crystal: 1746-01-6.01
23 - EPA Effluent Guidelines
32 - NIOSH RTECS: HP35000

Non-CIS References

34 - ORNL ETIC
77 - NLM CHEMLINE: TOXLINE, MEDLINE



Dibenzo[b,e][1,4]dioxin, 2,3,7,8-tetrachloro-(9CI)
Dibenzo-p-dioxin, 2,3,7,8-tetrachloro- (8CI)
Dioxin (herbicide contaminant)
TCDBD
TCDD
2,3,7,8-Tetrachlorodibenzo-p-dioxin
2,3,7,8-Tetrachlorodibenzo-1,4-dioxin

Figure 6. Name search for TCDD.

given in Figure 6. Here a search is made for the acronym TCDD. This is identified by the user as a complete or "whole" name and the system retrieves one compound, Registry number 1746-01-6, for which TCDD is a synonym. From the record for that compound, printed in Figure 6, it may be seen that use of either the 9CI or 8CI names presumes some familiarity with nomenclature rules. For this reason, it is important that name search programs be able to search for synonyms and name fragments in the manner shown.

Other Data Bases

As work proceeded on the development of the structure searching capability, new data bases were being added to the system. The biomedical research community had been supporting the development of search systems based on carbon-13 NMR (12) and x-ray diffraction data (13); at the same time, the environmental and regulatory community, particularly EPA and FDA, had taken the lead in the development of systems such as the Oil and Hazardous Materials Technical Assistance Data System, the Federal Register Search System (14), and the NIH-EPA Toxicology Search System (15), which is based on the NIOSH Registry of Toxic Effects of Chemical Substances (RTECS).

In each of these cases, it was necessary to be able to search the data base with numerical data. For example, just as a chemist might wish to identify all chemicals with a given NMR shift, a regulator may wish to learn of all chemicals that have been regulated by a specific agency or under a particular law. For this reason, each of these systems was equipped with its own search programs. Some programs were used in common by more than one system, but much of the code is, by its nature, unique to a single component. It is also necessary in every case, however, to be able to search the data base by structure, as was true with the mass spectral file. Rather than re-install the structure searching programs in each system, a single Structure and Nomenclature Search System was created which searches a single master file. This master file contains structure and nomenclature records for all chemical compounds in CIS. Every chemical carries in its record the identity of the files which have information on it. The files which are referred to in this way include all CIS data bases and also a wide variety of external files. Once a chemical is identified by SANSS, it is possible for the system to provide the Registry number of the compound, its molecular and structural formulas and chemical and trivial names and all files, CIS and other, which contain data on the compound. An example of this is shown in Figure 5.

Chemical Information System

The integrated Chemical Information System that emerged from these efforts is in fact a star-shaped network of search systems, as shown in Figure 7. The Structure and Nomenclature Search System is the "hub" or "center" of the network and provides name and structure search capability for any or all of the numeric data component files. Searching with numeric data, on the other hand, must be carried out in the appropriate component. Thus searching with mass spectral data as described in Figure 1 must be done in the Mass Spectral Search System. Compounds retrieved in this way are provided in the form of their CAS Registry numbers, and these can be used in SANSS to obtain the structures and names of the compounds or in MSSS to display the appropriate mass spectrum.

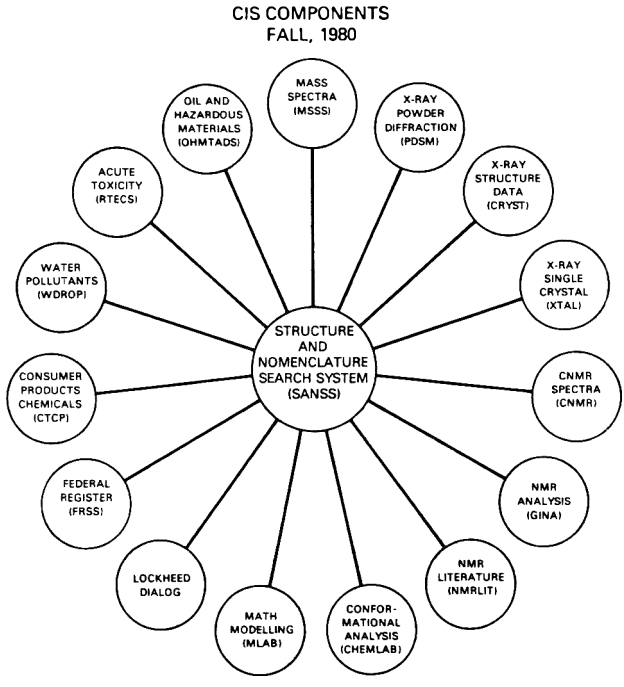


Figure 7. NIH-EPA Chemical Information System.

The possible uses of the entire CIS in either of these senses is depicted in Figure 8. As can be seen from this schematic diagram, any of a number of parameters such as name, structure, and molecular formula can be entered into SANSS. This returns Registry numbers which can be used to retrieve data such as spectra or toxicity information. Conversely, numeric data of this sort may be used to identify the Registry numbers of candidate compounds. The Registry numbers can then be used in turn to lead to chemical name and structure information. In this way, the CIS fulfills its goal of retrieving compounds on the basis of either structure or numeric data and is used frequently by those seeking to identify a compound from data and

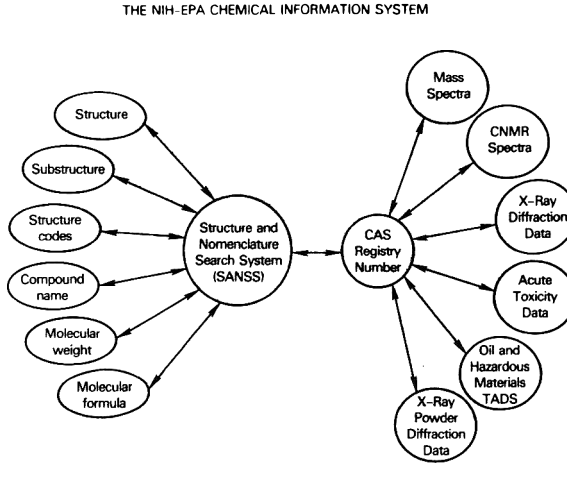


Figure 8. Structure and Nomenclature Search System (SANSS) and Chemical Locator Function (CLF).

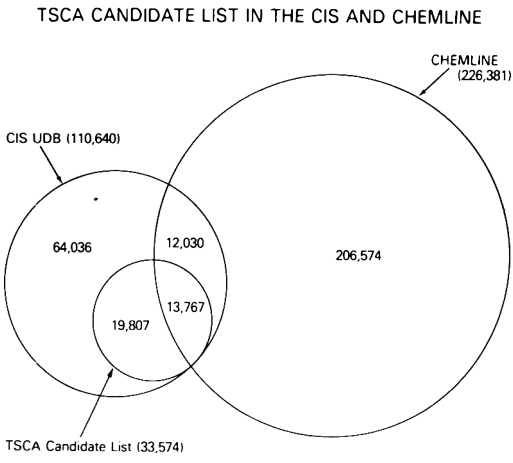


Figure 9. Chemicals common to TSCA Candidate List and CHEM-LINE or SANSS.

also those wishing to retrieve data pertaining to a specific compound.

File Management

SANSS is organized in terms of a number of files or "collections" of chemicals and each chemical is assigned its unique CAS Registry number. It is therefore possible to consider each file of chemicals as merely a list of Registry numbers; comparison of 2 such lists is, for a computer, a trivial exercise. When, in 1976, the TSCA Candidate List was assembled, it was quite simple to show the overlaps between this and CHEMLINE, NLM's chemical dictionary, and CIS/SANSS, as in Figure 9. Only 41% of the Candidate List appeared in CHEMLINE, while 100% of the list was in CIS/SANSS. In fact, the Candidate List, and later, the actual TSCA Inventory, were subsequently merged into both systems. In another application of this approach, the number of pesticides present in the EPA-FDA Collection of Reference Standards but absent from the mass spectral data base was established, as seen in Figure 10. Those missing from mass spectral data base were identified and samples of them were provided to a contractor who measured their mass spectra. The new spectra were added to the mass spectral data base which, as a result is more nearly exhaustive as far as registered pesticides are concerned. This should presumably make the file much more useful from the point of view of analysis of environmental samples.

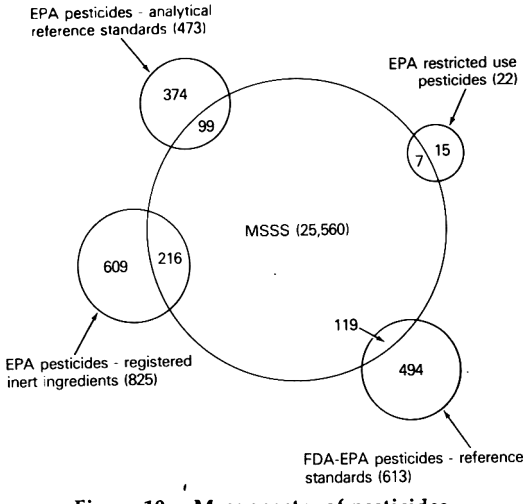


Figure 10. Mass spectra of pesticides.

Environmental Pollution

CIS is used extensively by EPA regional laboratories in dealing with chemical pollution. Depending on the problem, the agency uses various components of the system to retrieve and review data pertaining to identity, toxicity, regulation, and so on. In a typical example, EPA was called in to assist the State of Maryland in assessing the potential hazard associated with the cleanup of an abandoned tank farm in Sharptown, MD (16). Using gas chromatography-mass spectrometry (GC-MS) in conjunction with MSSS, agency scientists identified a number of organic materials present in the dump.

A typical contaminant was isophorone, whose SANSS record is given here shown in Figure 11. This revealed that there was a considerable amount of information available for the compound. It has an entry in RTECS, for example, review of which suggested that the compound is not highly toxic but is an eye irritant. Further exploration of CIS for entries dealing with isophorone provided information concerning the potential of the material on public health in case of a spill. Prior history as a water pollutant was found by using the *Distribution Register of Organic Pollutants in Water* and, finally, the regulatory status of isophorone was reviewed. Retrieval of all of these data for each compound required only a few minutes; when the task was completed, it was concluded that removal of the chemicals to a distant disposal site did not entail any intolerable risk. Accordingly, all the discarded chemicals were removed from the site and shipped uneventfully to a disposal facility in Baltimore, where they were destroyed.

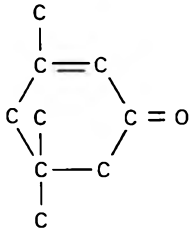
CAS RN 78-59-1

CIS Sources Of Information

CIS, EI Mass Spectrometry
CIS, Carbon-13 NMR Spectrometry: 78-59-1.01 to 78-59-1.03
EPA/CIS, OHM/TADS: 72T16766
NIOSH/CIS, RTECS: GW77000
EPA/CIS, WaterDROP

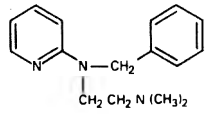
13 Non-CIS References Available

C9H14O

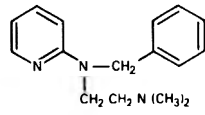


2-Cyclohexen-1-one, 3,5,5-trimethyl- (8CI,9CI)
.alpha.-Isophoron
.alpha.-Isophorone
Isoacetophorone
Isoforon
Isophoron
Isophorone
3,5,5-Trimethyl-2-cyclohexene-1-one
3,5,5-Trimethyl-2-cyclohexenone

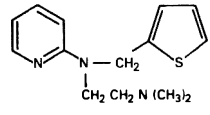
Figure 11. CLF record for isophorone.



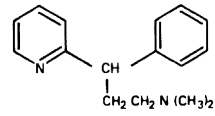
PYRIBENZAMINE
RN = 154-69-8



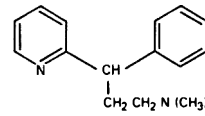
PYRILAMINE MALEATE
RN = 59-33-6



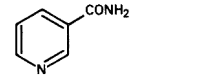
METHAPYRILENE
RN = 135-23-9



PHENIRAMINE
RN = 86-21-5



CHLOROPHENIRAMINE
RN = 113-92-8



NICOTINAMIDE
RN = 98-92-0

Figure 12. Derivative of pyridine found in decongestants.

Identification of Toxic Chemicals

Many products that can readily be purchased in the United States are simple mixtures of chemicals which are toxic alone or in mixtures. A wide variety of household products are in this category, and some of them are inevitably involved in cases of accidental intoxication. In an effort to ameliorate this problem, Hodge, Gosselin, and their coworkers have, over a number of years, compiled a data base (17) which contains the composition of many commercial products and the toxicity of the chemical constituents. This data base, the *Clinical Toxicology of Commercial Products*, has been installed in CIS. It is linked to SANSS, as described above, and the resulting system permits answering a variety of interesting questions.

A search for the entire SANSS data base for all compounds containing a pyridine ring leads to 6555 retrievals. If the CTCP file is examined to locate decongestants containing any of these compounds, a total of 127 currently available products are found, and further analysis of all the components of these 127 commercial products reveals that only 6 different pyridine derivatives are involved. Three of these, pyribenzamine, pyrilamine, and methapyrilene, are, as shown in Figure 12, in the pyribenzamine family. A further two, pheniramine and chloropheniramine, are congeners and the last one is nicotinamide.

An analysis of this sort shows how much may be learned from fragmentary evidence if a system such as CIS is available. In this fictional case, that the material was sold as a decongestant and that it contained a pyridine derivative were the only known facts, but they prompted a fairly exhaustive review of over-the-counter products which led to the reasonable conclusion that only a few such compounds are used in this way. Had this been an overdose case, an attending physician might be quite confident that one of these 6 compounds was involved in the patient's condition and treatment might be embarked upon with this in mind.

Applications of Artificial Intelligence

The most obvious shortcoming of a system, such as CIS, which seeks to identify chemicals by searching libraries of data is that if the unknown

Option? PEAK

Type peak, min int, max int
CR to exit, 1 for CAS RN, QI, MW, MF and Name

User: 85,100,100

File 1 contains 167 references to m/z 85

Next request: 100,0,10

File 2 contains 18 references to m/z 85 100

Next request: 128,0,10

File 3 contains 4 references to m/z 85 100 128

Next request: 198,0,10

There are no spectra in the file
with that combination of m/z peaks and intensities

Peak of 198 disregarded.

Next request: 1

How many (E to Exit)? 4

Type E to terminate display.

CAS RN	QI	MW	Formula, Names
104-61-0	656	156	C9H1602
			2(3H)-Furanone, dihydro-5-pentyl- (8CI,9CI)
			.delta.-n-Amylbutyrolactone
			.gamma.-n-Amylbutyrolactone
			.gamma.-Amyl-.gamma.-butyrolactone
			.gamma.-Amylbutyrolactone
104-67-6	649	184	C11H2002
			2(3H)-Furanone, 5-heptyldihydro- (8CI,9CI)
			.delta.-Undecalactone
			.gamma.-n-Heptylbutyrolactone
			.gamma.-Heptyl-.gamma.-butyrolactone
			.gamma.-Heptylbutyrolactone
105-21-5	531	128	C7H1202
			2(3H)-Furanone, dihydro-5-propyl- (8CI,9CI)
			.gamma.-Heptalactone
			.gamma.-Heptanolactone
			.gamma.-Propiobutyrolactone
			.gamma.-Propyl-.gamma.-butyrolactone
17160-89-3	686	128	C7H1202
			Ethanone, 1-(1-hydroxycyclopentyl)- (9CI)

Figure 13. Identification in MSSS of a beetle pheromone.

is not represented in the library, the approach will fail. In practice, however, it is often possible in cases of this sort to extract considerable information from the data bank and use it to complete the identification of the unknown material. The trial pheromone from a beetle (18), after GC-MS, was shown to have a major component with prominent mass spectral peaks at m/z 85, 100, 128, and 198. When these data were used in an MSSS search as shown in Figure 13, 4 compounds were found with peaks at m/z 85, 100, and 128, but none of these also had a peak at m/z 198. Review of the structures of the 4 retrieved compounds, however, showed that 3 were all gamma-lactones with side chains of varying length. The gamma-lactone derived from 3-hydroxy-*n*-dodecanoic acid would have a molecular weight of 198 and should give peaks at m/z 85, 100, and 128, in its mass spectrum. This, therefore, is a logical candidate for the pheromone, and subsequent synthesis and testing of the material showed that it is, in fact, the correct compound.

Subsequently, attempts have been made to systematize approaches of this sort to problems in structure determination. The general technique has been to use statistical analysis of the mass spectral file in an effort to identify rules which can reliably lead to structural information. Thus a number of rules were established in this way which permit a determination as to whether or not the molecule whose mass spectrum is at hand contains chlorine (19). In the spectrum shown in Figure 14, the program first attempts an estimate of the molecular weight of the unknown. It is at once deduced that neither the base peak at m/z 99 nor the minor ion at m/z 105 can be the molecular ion because if either is the molecular ion, the other is inexplicable. In fact, the program concludes that 134 is the most probable molecular weight and if that is correct, the probability is 100% that the molecule contains chlorine. It then proceeds to present other, less likely combinations of molecular weight and probability of presence of chlorine but, as can be seen from Figure 14, the compound in question is 1-chloro-4-ethoxy-but-2-ene, with a molecular weight of 134, just as the program forecast.

CAS RN	QI	MW	MF, Name
53957-23-6	664	134	C6H11C10
			2-Butene, 1-chloro-4-ethoxy- (9CI)

Instrument ATLAS CH4, Inlet ,Source temp. 270 C, ev. 70
Contributor R. F. HUDSON UNIVERSITY OF KENT, CANTERBURY

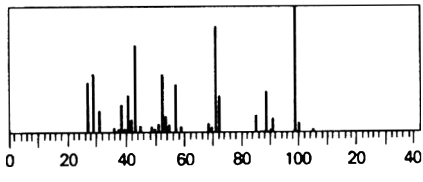


Figure 14. Mass spectrum of 1-chloro-4-ethoxy-but-2-ene.

Detection of the presence of chlorine in a molecule from its mass spectrum is relatively simple in some 90% of all spectra but in the remaining 10%, the difficulty is considerable. This program appears to handle many of the difficult spectra, and over the whole data base it reaches the correct result in about 96% of all cases. Perhaps the most interesting facet of this approach is that it is quite general. Because of this it has proved possible, by using the same approach, to generate rules which permit determination of the presence or absence in a molecule of bromine, nitrogen, and a phenyl ring.

Use of CIS

As has been mentioned previously, the entire CIS resides on computers in the private sector where it is publicly accessible on a fee-for-service basis.[1] The U.S. government does not subsidize the use of the system and, indeed, must pay to use it just like any other user. There is continuing government support for various aspects of the development of the system. In the past, much of this support went for software development. Currently, however, the CIS software is fairly stable and a larger proportion of the government support is used for data collection and validation.

The computers used by CIS have, since 1972, been linked to international networks such as GE, Tymnet, and GTE-Telenet. As a result, there is a considerable amount of use of CIS from over 20 countries other than the United States. About 25% of all CIS usage originates from countries other than the United States.

About 30 000 transactions per month are now logged by the system and the number of users is approximately 1000. The cost of using CIS depends on which component is being used and varies between $45 and $90 per connect hour. The time required for a search in CIS may be anywhere between 1 and 10 min, and the costs therefore lie between $1 and $15. Network and, in countries outside the United States, PTT charges, are additional. The network charges are on the order of $10 per hour and the PTT charges, which vary with country, are on the same order.

[1] To obtain access to CIS, please contact CIS Inc., 7215 York Rd, Baltimore, MD 21212; telephone (301) 821-5980.

References

(1) Heller, S. R. (1972) *Anal. Chem.* **44,** 1951–1961; Heller, S. R., Fales, H. M., & Milne, G. W. A. (1973) *Org. Mass Spectrom.* **7,** 107–115; Heller, S. R., Fales, H. M., & Milne, G. W. A. (1972) *J. Chem. Ed.* **49,** 725

(2) Hertz, H. S., Hites, R. A., & Biemann, K. (1971) *Anal. Chem.* **43,** 681–690

(3) Pesyna, G. M., Venkataraghavan, R., Dayringer, H. E., & McLafferty, F. W. (1976) *Anal. Chem.* **48,** 1362–1368

(4) Feldmann, R. J., & Heller, S. R. (1972) *J. Chem. Doc.* **12,** 48–54

(5) Lynch, M. F., Harrison, J. M., Town, W. G., & Ash, J. E. (1973) *Computer Handling of Chemical Structure Information*, Macdonald, London, UK

(6) Feldmann, R. J. (1974) in *Computer Representation and Manipulation of Chemical Information*, S. R. Heller, W. T. Wipke, R. J. Feldmann, & E. Hyde (Eds), John Wiley & Sons, Inc., New York, NY, pp. 55–81

(7) Feldmann, R. J., Milne, G. W. A., Heller, S. R., Fein, A. E., Miller, J. A., & Koch, B. (1977) *J. Chem. Inf. Comput. Sci.* **17,** 157–163

(8) Heller, S. R., & Milne, G. W. A. (1980) *J. Chem. Inf. Comput. Sci.* **20,** 204–211

(9) Heller, S. R., Milne, G. W. A., & Feldmann, R. J. (1976) *J. Chem. Inf. Comput. Sci.* **16,** 232–233

(10) Milne, G. W. A., Heller, S. R., Fein, A. E., Frees, E. F., Marquart, R. G., McGill, J. A., Miller, J. A., & Spiers, D. S. (1978) *J. Chem. Inf. Comput. Sci.* **18,** 181–186

(11) Heller, S. R., & Milne, G. W. A. (1980) *Anal. Chim. Acta* **122,** 117–138

(12) Dalrymple, D., Milne, G. W. A., Wilkins, C. L., & Heller, S. R. (1978) *Org. Magn. Reson.* **11,** 535–540; Zupan, J., Heller, S. R., Milne, G. W. A., & Miller, J. A. (1978) *Anal. Chim. Acta* **103,** 141–149; Milne, G. W. A., Zupan, J., Heller, S. R., & Miller, J. A. (1979) *Org. Magn. Reson.* **12,** 289–296

(13) Marquart, R. G., Katsnelson, I., Milne, G. W. A., Heller, S. R., Johnson, G. G., & Jenkins, R. (1979) *J. Appl. Crystallogr.* **12,** 629–634

(14) Marquart, R. G., Marquart, L., Mintz, S., McGill, J. R., McDaniel, J., Heller, S. R., & Milne, G. W. A. (1980) *Online* **4,** 45–49

(15) McGill, J. R., Heller, S. R., & Milne, G. W. A. (1978) *J. Environ. Pathol. Toxicol.* **2,** 539–551

(16) Milne, G. W. A., Fisk, C. L., Heller, S. R., & Potenzone, R. (1982) *Science* **215,** 371–375

(17) Gosselin, R. E., Hodge, H. C., Smith, R. P., & Gleason, M. N. (1975) *The Clinical Toxicology of Commercial Products*, 4th Ed., Williams & Wilkins, Baltimore, MD

(18) Wheeler, J. W., Happ, G. M., Araujo, J., & Pasteels, J. M. (1972) *Tetrahedron Lett.* **46,** 4635–4638

(19) Meisel, W., Jolley, M., Heller, S. R., & Milne, G. W. A. (1979) *Anal. Chim. Acta* (1979) **112,** 407–416

Interfacing an ICAP System to Food and Drug Administration's Large Computer Facility

STEPHEN G. CAPAR and LAURENCE R. DUSOLD[1]
Food and Drug Administration, Division of Chemical Technology, Washington, DC 20204

An inductively coupled argon plasma (ICAP) emission spectrometer is capable of simultaneously determining the concentrations of many elements. The Jarrell-Ash ICAP systems at FDA have dedicated minicomputers which control the analysis via terminal commands. The standard ICAP terminal interface has been changed to enable logging of ICAP data onto a magnetic tape data cartridge. This tape is subsequently processed through the programming language APL into a large computer system. The transfer of data into the large computer system enables additional statistical processing, report writing, archival storage, and magnetic reel tape generation for transferring processed data to computer systems of other agencies. Three of FDA's ICAP systems use these data processing procedures. The ICAP terminals have been upgraded or replaced from current loop 110-300 baud to RS-232-C 1200 baud and equipped with magnetic tape data cartridge units. Separate graphics display terminals with magnetic tape data cartridge units are used to read the data into the large computer system at 1200 or 4800 baud.

The Food and Drug Administration (FDA) has a number of minicomputer-controlled inductively coupled argon plasma (ICAP) emission spectrometers which perform simultaneous multi-element determinations. Most of these instruments provide $\mu g/mL$ or $\mu g/g$ results in less than 1 min. The instruments are primarily used for studies in which a large number of samples must be analyzed for many elements. Examples of such studies are surveys of processed foods or raw agricultural products, research on new analytical methods, and tissue analysis of animals involved in nutritional or toxicological feeding studies. The ICAP multi-element capabilities are also used to investigate a product suspected of containing dangerously high levels of an element (such as lead or cadmium).

The large amount of data generated by the ICAP is usually statistically evaluated and condensed before final reporting. The statistical analysis (i.e., correlation, frequency plot, and/or percentile) is performed on a large IBM 3033 computer system. Older model ICAPs provide results only in units of $\mu g/mL$. These results must be further processed to obtain a $\mu g/g$ result. This relatively simple calculation becomes very tedious when it must be done for all the elemental results of each sample. The large computer system could perform this computation very efficiently. Therefore, it was advantageous to enter the data into the computer system to perform the simple yet redundant calculations and still have access to many different statistical routines. Because of the time involved and the potential for errors, keying the data into the computer system was to be avoided.

FDA's first ICAP system used a Teletype ASR 38 terminal with paper tape reader/punch to enter the ICAP operating programs. By activating the paper tape punch while the ICAP results were printing on the terminal, a computer-readable record was produced. The paper tape record was then read into our large computer system via the programming language APL (1). For convenience, the paper tape was copied onto a magnetic tape data cartridge before it was processed on the large computer. The problems inherent with paper tape were overcome by replacing the reader/punch with a magnetic tape data cartridge. This device was also incorporated into our other 2 ICAP systems, which used floppy disks instead of paper tape to enter and store ICAP operating programs. At the same time, the ICAP terminals were upgraded from current loop 110–300 baud to RS-232-C 1200 baud. The ICAP system whose paper tape reader/punch was replaced also had to be upgraded to a floppy disk system so that the ICAP operating programs could be entered and stored. This totally eliminated use of paper tape for either input or output and made the entire ICAP system approximately 10 times faster than that originally supplied by the vendor.

The ICAP Instrument

Three of FDA's ICAP systems have been upgraded to high speed terminals and magnetic tape data cartridge logging devices. Two of these ICAPs are located in Washington, DC and one is in Cincinnati, OH. All 3 instruments are Jarrell-Ash Model 975 Plasma Atom Comp, 0.75

Presented at the Symposium on Computers in the Laboratory, 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

[1] Division of Chemistry and Physics.

0004-5756/82/6505-1259-05$01.00

```
EGGAC

BURN # 1   FDA1        9:06    22JUL81

BURN # 2   FDA1        9:06    22JUL81

AVERAGE    FDA1        9:07    22JUL81
   IS
 2843
   AL      AS       B        BE       CA       CA       CD       CO       CR       CU
 .1526   .0584   .3412 <      0    24.74    25.88 <      0   .0079   .0072 <      0
   FE      GE       MG       MN       MO       NI       NI        P       PB       SB
9.907   .0532   19.53    .1580    .0302    .0376    .0459    37.39 <      0 <      0
   SE      SN       TE       TI       TL        V       ZN        K
<    0  .0585   .2183    .0088    .0523 <      0   .2380   129.3
```

Figure 1. Output from ICAP with PDP-8 minicomputer. Elemental results in units of μg/mL.

m direct reading spectrometers. They incorporate dedicated Digital Equipment Corp. (DEC) minicomputers which control the analysis via terminal commands. Two of the ICAPs have PDP-8/E or PDP-8/M minicomputers, whereas the third ICAP has a more powerful PDP-11 minicomputer. Each of the ICAPs is capable of determining about 30 elements simultaneously. The minicomputer performs the quantitation by first preprogramming the concentration of a set of standard solutions. The solutions are then analyzed to obtain and store the response measurements. Two different concentrations for each element are used for calibration. Linearity is assumed. Sample solution responses are compared to the calibration data to obtain element concentrations.

The 2 PDP-8 ICAP systems report results in units of μg/mL. The PDP-11 ICAP system uses a dilution factor obtained from the entered weight and volume of a sample to compute μg/g results. All 3 systems allow comment statements to be typed at the ICAP terminal. This comment facility is used to flag information to be processed by an APL program. An example of the output from an ICAP with a PDP-8 minicomputer is given in Figure 1.

Different types of solutions are analyzed, such as sample, reagent blank, and quality control solutions. The sample and quality control solutions must be corrected for the concentration of the elements in their respective reagent blank solutions. This correction is automatically performed by the ICAP with the PDP-11. The other ICAPs, however, require the correction to be made apart from the ICAP system.

ICAP Hardware Interface

A diagram of the hardware interface of the high speed printer and data cartridge is given in Figure 2. The following hardware is required but was not part of the original ICAP system.

(a) *Printer terminal.*—Capable of 1200 baud RS-232-C (DEC LS-120 HE, or LA-120 with RS-232-C, or equivalent). This replaced a teletype

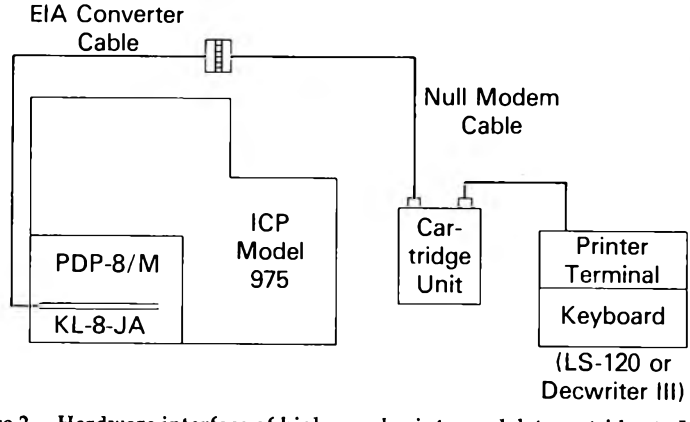


Figure 2. Hardware interface of high speed printer and data cartridge to ICAP.

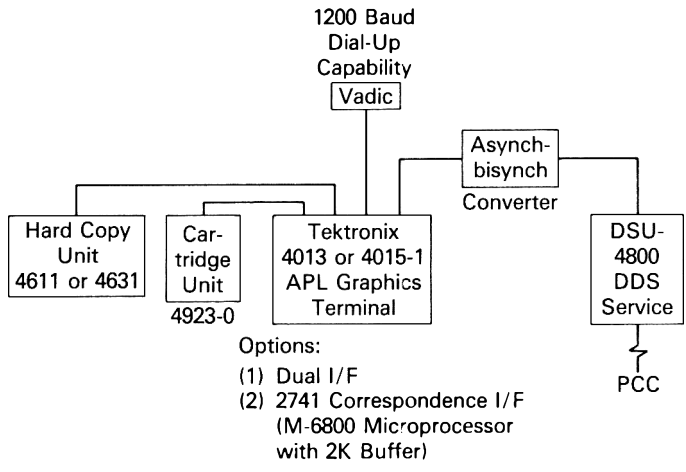


Figure 3. Configuration of communication equipment to IBM computer.

ASR-33 on one of our instruments and a LA-36 on the others.

(b) *Data cartridge unit.*—Capable of line connection. A Tektronix No. 4923-01 was used with DC 300A certified tapes.

(c) *Terminal interface board.*—The DEC PDP-8/M required that its KL8-E asynchronous data control board be replaced with a KL8JA asynchronous data I/O board. The PDP-8/E already had a KL8JA board. The PDP-11 had its asynchronous data control board replaced with a DL11WB asynchronous data I/O board.

(d) *Cables.*—Null modem (DEC No. BC03M-25) and EIA converter cable (DEC No. BC01V-25).

There are 5 functions on the 4923-01 Data Cartridge Unit: on-line, reverse, write, run, and forward. In order for the terminal to interact with the ICAP, the power switch of the cartridge unit must be on and the "on-line" switch must be depressed. When no other switches are activated, the data cartridge unit is transparent to the system. When the "on-line" and "write" switches are activated, the data cartridge unit records all information passing between the terminal and the ICAP. The information will still be printed at the terminal. Data already recorded on a cartridge can be printed at the terminal by turning the "on-line" switch off and activating the "run" switch.

Interfaces

Large Computer Interface

All 3 ICAP facilities have equipment available to access FDA's IBM 3033 computer system located at the Parklawn Computer Center in Rockville, MD. Two of the ICAP facilities have this equipment in the laboratory near the ICAP. The equipment includes a Tektronix 4013 or 4015-1 graphics display terminal with the APL character set, a Tektronix 4923-0 data cartridge unit, and a Tektronix 4610 or 4631 hard copy unit. Telecommunications for the terminals are provided by either a Bell 212A modem used at 1200 baud or Bell 500A with Dataphone Digital Service at 4800 baud. Figure 3 shows the configuration of this equipment. The equipment is not dedicated to ICAP data reduction but is used by a number of chemists for other projects. The ICAP data reduction is performed by the chemist, providing the analyst with the opportunity to review the data and ensure its integrity.

ICAP Software Interface

Elemental data can be obtained for different types of solutions such as a sample, sample reagent blank, quality control, calibration standard, or standard reagent blank. Not all information coming from the ICAP is useful; therefore, once information to be processed by the large computer is distinctly identified, all the excess data can be ignored simply by using the comment statement with keywords. A list of keywords used with the PDP-8 ICAPs is given in Table 1. The keyword identifies the ICAP data that are next to the keyword or that immediately follow it in a fixed sequence. The weight and volume of the sample are also entered; keywords are used for the 2 ICAPs which provide only μg/mL results. The location of the elemental results is identified by the first elemental symbol. The rigid structure of the ICAP output enables all the elemental results to be located in relation to the

Table 1. ICAP keyword phrases

Keyword	Definition
*/ID=	Characters after "=" are sample identification. Next ICAP elemental data is analysis of sample. Weight and volume data expected before elemental data.
*/WGT=	Sample weight follows "=".
*/VOL=	Sample volume follows "=".
XX	Elemental data are on next few lines (number of lines depends on ICAP system). XX is symbol of first element listed by ICAP.
*/BLK	Elemental data that follow are for sample reagent blank.
*/STD	Elemental data that follow are for standard
*/ID=	solution. Characters after "=" are standard identification.
*/STD	Elemental data that follow are for standard
*/BLK	reagent blank.
*/END	End of analytical session.

first line of elemental symbols. An example of a sample and standard analytical printout from a PDP-8 ICAP is given in Figure 4. The PDP-11 ICAP, which provides $\mu g/g$ results, also lists the weight and volume of the sample. These data are presented in a strict format and can be easily located for use by the large computer.

APL Software Interface

The ICAP data are made available to the large computer system through the interactive computer language APL (2). Our computer system runs APL.SV version 3.1 in a time sharing environment under the operating system MVS/SE2 Release 3.8. Programs have been written in APL which perform a variety of functions (1). The

```
*/ID=VEGETABLE JUICE 41

*/VOL= 50

*/WGT=10.00

*O
EGGAC

BURN # 1  FDA1      9:06   22JUL81

BURN # 2  FDA1      9:06   22JUL81

AVERAGE   FDA1      9:07   22JUL81
   IS
  2843
   AL      AS      B       BE      CA      CA      CD      CO      CR      CU
 .1526   .0584   .3412 <     0   24.74   25.88 <     0   .0079   .0072 <     0
   FE      GE      MG      MN      MO      NI      NI      P       PB      SB
 9.907   .0532   19.53   .1580   .0302   .0376   .0459   37.39 <     0 <     0
   SE      SN      TE      TI      TL      V       ZN      K
<    0   .0585   .2183   .0088   .0523 <     0   .2380   129.3


*/STD

*/ID= QC37

*O
EGGAC

BURN # 1  FDA1      9:08   22JUL81

BURN # 2  FDA1      9:09   22JUL81

AVERAGE   FDA1      9:09   22JUL81
   IS
  2841
   AL      AS      B       BE      CA      CA      CD      CO      CR      CU
 14.66   .2178   5.026 <     0   4.913   5.200   1.008   .0001   .0334   5.128
   FE      GE      MG      MN      MO      NI      NI      P       PB      SB
<    0   5.226   .0023   .0010   .0087   1.024   .9968   .1361 <     0 <     0
   SE      SN      TE      TI      TL      V       ZN      K
 .0305   5.082 <     0   .0005   5.132 <     0   5.009 <     0
```

Figure 4. Output from ICAP with PDP-8 minicomputer including sample and standard identification.

initial program used on an ICAP data cartridge translates the upper case ASCII ICAP characters into the appropriate APL characters. These translated raw data are temporarily stored in an N × 80 matrix, which is an image of the ICAP terminal output (excluding blank lines). This matrix is then sequentially scanned for keywords that cause the appropriate action to be taken on the data. The elemental results, identifications, weights, volumes, etc., are all stored in individual variables to await further processing. The time and date stamp of each analysis is also saved. The $\mu g/g$ calculation can now be easily performed in the APL workspace. At this point, the data are available for any APL program supported by the computer center or written by an individual. The data can also be transferred to computer disk files and then through APL batch submit to be used by other programs supported by the computer facility.

Benefits of Large Computer Systems

Having our data on a large computer system in APL has many added advantages. Tabular listing of analytical results in a variety of formats is available through programs written in APL. This output can be produced immediately on the graphics display terminal or, through the aid of disk file storage, on either microfiche or a high speed line printer. Both the microfiche and high speed line printer can furnish multiple copies. We are also able to produce microfiche of the unprocessed ICAP data. This medium takes up much less space for archival storage but still fulfills the requirements of the federal regulations on Good Laboratory Practices (3), which require that duplicate copies of all analytical data be maintained for a number of years.

Selected ICAP data can be put on disk files through APL and submitted to system-supported statistical packages such as SAS (Statistical Analysis System, SAS Institute, Inc.). Changes can be made to the files as well as additions of elemental data from sources other than the ICAP (i.e., atomic absorption spectroscopy or anodic stripping voltammetry).

Conclusion

The integration of the ICAP system to a large computer facility has been extremely successful in terms of efficiency and versatility. The relatively inexpensive and simple interface makes it possible to take full advantage of data reduction routines available on a large computer system. The APL language has proved to be fully capable of managing the ICAP data. The nature of this computer language has permitted the rapid development of specifically requested data reduction procedures. The data logging and computer processing of ICAP data has become standard practice for all 3 FDA ICAP facilities.

References

(1) Capar, S. G., & Dusold, L. R. (1978) *Am. Lab.* **10**, 17–28 *passim*

(2) Falkoff, A. D., & Iverson, K. E. (1973) *IBM J. Res. Dev.* **17**, 324–334

(3) *Code of Federal Regulations* (1981) Title 21, Part 58, Subpart J

Computer Utilization in the Food and Drug Administration's Bureau of Foods Mass Spectrometry Laboratory

PETER A. DREIFUSS and LAURENCE R. DUSOLD
Food and Drug Administration, Division of Chemistry and Physics, Washington, DC 20204

A network of computers is being used to support the Food and Drug Administration's Bureau of Foods mass spectrometry facility. Five mass spectrometers are each interfaced to at least 2 of the 6 dedicated minicomputers in the laboratory. This multiple interfacing provides data acquisition and processing backup, reducing the overall down-time. Selected data from all of the minicomputers can be sent to FDA's main computers via a digital cartridge tape recorder or paper tape. The digital cartridge tape recorder records data that are output from a minicomputer terminal and then plays it back on a terminal which is on-line with the main computer. This main computer stores and edits data; plots spectra for reports, data banks, and publications; and carries out some data processing. Multiple interfacing also serves to supplement the capabilities of the 8-year-old Finnigan MAT (formerly Varian MAT) SS-100 data system (Sperry-Univac/V-76) with the newer and more powerful Finnigan MAT INCOS (Data General/Nova 3) data system. The SS-100 data system is also enhanced by the substitution of the 110 baud paper tape with a 9600 baud cartridge tape recorder for I/O of system bootstraps, BASIC programs, and raw data.

The advance of computers and the development of mass spectral software have contributed much to mass spectrometry (MS) in the last decade (1). The dedicated minicomputer has made it possible to manage the extremely large quantity of MS data which can be generated. This ability has proved to be especially valuable for the analysis of complex mixtures by gas chromatography (GC)/MS. The much larger time-shared computer systems run user-written programs, store MS data, and search large libraries of MS data.

The Food and Drug Administration's Bureau of Foods (BF) MS laboratory first used a digital device for the acquisition of data in 1971. An Infotronix digital data acquisition device was used to acquire time/intensity data and record it on magnetic tape. This magnetic tape was then read by the FDA central computer, which could recreate the mass spectrum. Since that time, mass spectrometer and computer instrumentation has increased from 2 mass spectrometers and the Infotronix device to 7 mass spectrometers, 7 mini-computers, and additional terminals for communication with much larger time-shared computer systems. The intention of this paper is to describe the network of computers which has been implemented in the BF MS laboratory and the enhancements that have been made, which broaden the field of MS applications but minimize mass spectrometer dependence on any single data system.

Mass Spectrometry Computer Network

Table 1 lists the data systems currently in use in the BF MS laboratory and the mass spectrometers to which each is interfaced. Pertinent hardware and the means employed to transmit data to FDA's central computer system are also included. Not included in the table is a Hewlett-Packard 9825A calculator with dual floppy discs, which is an inherent part of a Hewlett-Packard 5992A GC/MS system and therefore not a part of the network of computers described below.

Figure 1 depicts the organization of the BF MS computer network. The network is mainly comprised of the 6 data systems described in Table 1 which are interfaced to 6 mass spectrometers. In this network, 5 of the mass spectrometers are each interfaced or can be interfaced to at least 2 data systems. This multiple interfacing minimizes mass spectrometer dependence on any single data system. It allows an alternative data system for 5 of the mass spectrometers if the data system normally used should require service or periodic maintenance. It also permits an operator to choose the data system which will provide the best software for a particular analysis. The Finnigan MAT 1015SL (purchased 1970) is currently interfaced to only one data system. An alternative data system was deemed to be unnecessary because of its limited use.

FDA's large central computer facility, known as the Parklawn Computer Center (PCC), consists of two IBM 3033 computers. As shown in Figure 1, it is also considered part of the computer network. Data from any minicomputer in the laboratory can be easily transferred to PCC. Two Tektronix Model 4923-0 digital cartridge tape

Presented at the Symposium on Computers in the Laboratory, 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

0004-5756/82/6505-1264-04$01.00

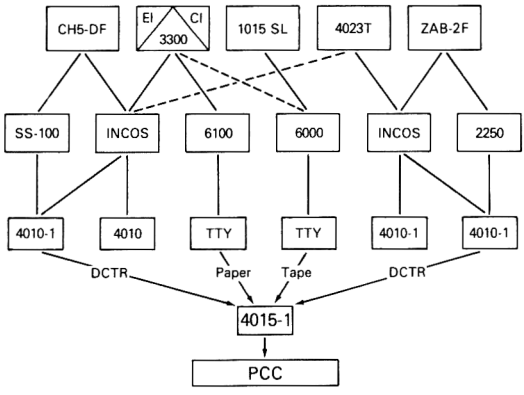


Figure 1. Computer hardware interconnection and data transmission routes in BF MS network.

Solid lines depict connections most frequently used. Broken lines indicate that a cable must be moved from its normal position to establish connection. The 3300 CI and EI are actually 2 independent mass spectrometers. The top row are all mass spectrometers. The second row are minicomputer data systems. The third row are all computer terminals: Tektronix Models 4010-1, Tektronix Model 4010, and ASR33 teletypes (TTY). The respective interfaces to the Tektronix Model 4015-1 terminal and PCC are depicted at bottom.

recorders (DCTRs) are used to record ASCII representation of MS data from 2 Tektronix 4010-1 terminals at 9600 baud. Data can be obtained in this fashion from 4 minicomputers. Data from the Finnigan MAT 6000 and 6100 computers are punched on paper tape through 110 baud ASR33 teletypes. A Tektronix 4015-1 terminal, which can communicate directly with PCC, is interfaced to both a DCTR and an optical paper tape reader/perforator. By using these 4015-1 terminal peripheral devices, the minicomputer data recorded on DCTR tape cartridge or paper tape can be transmitted to PCC at 4800 and 2000 baud, respectively. Currently this method is used to transmit individual spectra for storage or graphics plotting.

Network Components

The Finnigan MAT (formerly Varian MAT) SS-100 data system is used only with the Finnigan MAT CH5-DF mass spectrometer. It has the standard capabilities of other data systems but lacks certain desirable features of software (mass spectral addition, deconvolution of GC/MS peaks, real time data processing) and hardware (automatic operating system reloading). We have compensated for the lack of sophistication of the SS-100 data system by enhancing the performance with a DCTR or by using the INCOS data system as an alternative when required.

A DCTR interfaced into the minibus of the Tektronix 4010-1 terminal resulted in the complete elimination of the paper tapes normally employed for system reloading and maintenance, and for reading in user-written BASIC routines such as mass spectral addition (2). Bootstraps and programs are read in from a DC-300A or DC-300XL data cartridge at 9600 baud instead of 110 baud from a teletype paper tape reader. These procedures only require a few seconds at this higher speed.

The DCTR interfaced to this terminal is also used for data transfer of one spectrum at a time to and from PCC, as described above. One wiring modification was made to the terminal to allow switch-selectable insertion of mark parity regardless of the parity on the tape. Mark parity is required only for the re-entry of spectral data to the SS-100 data system.

An INCOS 2300 data system was purchased in 1978 primarily for the acquisition of data from the Finnigan MAT 3300 electron ionization (EI) and/or chemical ionization (CI) mass spectrometers. The INCOS could be interfaced to 2 mass spectrometers and 2 terminals simultaneously. This permitted an interface to the CH5-DF and the Tektronix 4010-1 terminal normally used with the CH5-DF/SS-100 system. However, to permit the terminal to be used with both data systems without changing cables and strappable options in the terminal each time, several hardware modifications were made (see Figure 2). First, a T-bar switch was installed to permit simple data system selection. Second, since the SS-100 and INCOS data systems use opposite data transmission formats, the modem/computer strappable option connection was changed from SS-100 orientation (direct computer connection) to the INCOS orientation (modem connection-normal position (3). Third, the SS-100 data transmission format was re-established at an RS-232 connection between the T-bar switch and the SS-100. The wires on pins 2 and 3 were interchanged and the wire on pin 20 was relocated to pin 5. Fourth, since the INCOS data system required a carriage return graphic input terminator, the graphic input terminator strappable option on card TC-2 was changed to the middle position (carriage return only). These modifications allowed both data systems to perform normally with a Tektronix 4010-1 terminal equipped for use with an SS-100 data system.

The state-of-the-art software of the INCOS 2300 has been beneficial for data acquisition and

Table 1. Data systems in Bureau of Foods MS facility

System	Vendor	CPU	Memory, bytes	Peripheral disc drive	Disc storage capacity, bytes	Data output to IBM via	Interfaced to
INCOS 2300	Finnigan MAT	Data General Nova 3/12	64K	CDC Model 9448 (Phoenix type disc)	32M	DCTR	Finnigan MAT 3300EI Finnigan MAT 3300CI Finnigan MAT CH5DF Finnigan MAT 4023T
INCOS 2300	Finnigan MAT	Data General Nova 3/12	64K	CDC Model 9448 (Phoenix type disc)	32M	DCTR	Finnigan MAT 4023T VG Analytical ZAB-2F
SS-100	Finnigan MAT	Sperry-Univac V-76	128K (32K in use)	CDC Model 9427H (IBM 5440 type disc)	12.5M	DCTR	Finnigan MAT CH5DF
2250	VG Analytical	DEC PDP 8A (2)	48K + 48K	Systems Industries (IBM 5440 type disc)	12.5M	DCTR	VG Analytical ZAB-2F
6100	Finnigan MAT	Computer Automation Alpha 16	32K	Diablo Model 31	3M	paper tape	Finnigan MAT 3300EI Finnigan MAT 3300CI
6000	Finnigan MAT	Computer Automation Alpha 16	32K	Diablo Model 31	3M	paper tape	Finnigan MAT 1015SL Finnigan MAT 3300EI Finnigan MAT 3300CI

processing with the Finnigan MAT 3300 EI, 3300 CI, and CH5-DF mass spectrometers. Switching INCOS data acquisition between the 3300 EI, 3300 positive ion CI, or 3300 negative ion CI involves the connection of the appropriate external mass set and signal cables to the INCOS. The 3300 CI is modified to perform pulsed positive negative ion chemical ionization MS (4). With this dual acquisition, no other acquisition may take place simultaneously, because both interfaces are used. However, data from the 3300 EI and CH5-DF may be acquired and processed on the Finnigan MAT 6100 (or 6000) and the SS-100 data systems, respectively. Data acquired from the 6000 or 6100 data systems may be transferred to PCC by paper tape as described above. Data acquired on the INCOS data system may be transferred to PCC by displaying the data of interest on the 4010-1 terminal interfaced to the DCTR and recording the data on the tape cartridge. These data can then be transferred to PCC in the same fashion as described for the SS-100 data.

A Tektronix dual interface option is installed on the Tektronix 4010-1 terminal, which is used primarily for the ZAB-2F mass spectrometer. With 2 external switches on the dual interface of the terminal, the operator can switch-select between the 2250 or INCOS data system. A 4923-0 DCTR, which is interfaced to the 4010-1 terminal, can record single spectra from either data system. These data can then be transmitted to PCC for plotting or storage as described previously.

PCC Interface

Adjacent to the MS laboratory is a complete graphics terminal installation with a 4800 baud Dataphone Digital Service hardwired to PCC. The graphics terminal hardware consists of a Tektronix 4015-1 terminal with enhanced graphics, dual interface, M-6800 microprocessor with 2K RAM buffer, 4923-0 DCTR, 4662-AA plotter with 8K buffer option, 4911 high speed reader/perforator for paper tape, and a 4631 hard copy unit. The dual interface allows 1200 baud communication with a Bell 212-A Dataphone in addition to the 4800 baud line to PCC. This permits easy access to the NIH/EPA Chemical Information System (5). Searches may be performed by using the Mass Spectrometry Search System option of the Chemical Information System to aid in the interpretation of an unknown compound.

The procedure to eliminate paper tape bootstraps with the SS-100 data system involved reading the paper tape bootstraps with the 4911 optical paper tape reader/punch. This was done in an "off-line" mode, and a data cartridge copy

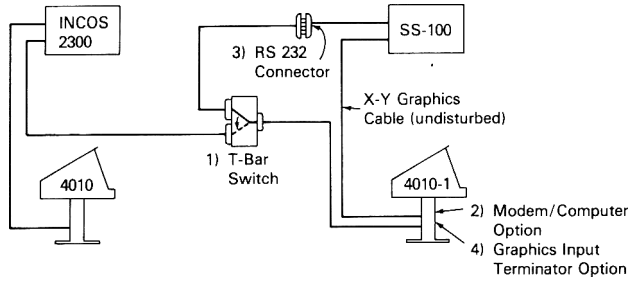


Figure 2. Hardware modifications to allow Tektronix Model 4010-1 terminal communication to Finnigan MAT SS-100 and INCOS 2300 data systems.

of the paper tape bootstraps was made. In this manner bootstraps for memory reload, system generation, and certain diagnostic programs were recorded. This process had to be done only once. All of the bootstraps were separated by file marks on the tape cartridge, so that the correct one could easily be selected.

The DCTR of the 4015-1 terminal is used as a data transfer medium between the MS laboratory and PCC. A data cartridge with data from the SS-100, 2250, or the 2 INCOS data systems is then physically transferred to the 4015-1 terminal's DCTR and read into an APL program at PCC. Paper tapes of MS data from the 6000 and 6100 data systems are read at 2000 baud with the 4911 reader/perforator on-line to an APL program at PCC. The program reformats data from paper tape or tape cartridge so that they can be replotted in one of many formats to produce high quality ink plots of the mass spectra. If the data, for example, concerned a mycotoxin, they could be added to the data base of over 100 mycotoxins available "on line" in APL at PCC (2, 6). Other data bases of interest to FDA are presently under development. All of the data bases use APL software to permit search by name or molecular weight.

Summary

The use of computers in the BF MS laboratory is designed to maximize the benefits derived from computers and minimize dependence on any single computer. A mass spectrometer interfaced to a single data system effectively suffers costly down-time when the data system is inoperable. The BF MS laboratory network of data systems is capable of significantly reducing overall instrumental down-time by providing data acquisition and processing backup. It also provides the analyst the option to choose the best data system for a particular analysis.

DCTRs provide a rapid method to transfer data from a minicomputer to another computer. They are presently used in the BF MS laboratory to transfer data to the IBM 3033 computers for storing and editing data and creating graphics reproductions. They are also used to completely replace the much slower paper tapes on the Finnigan MAT SS-100 data system.

The computer network of the BF MS laboratory has evolved to improve the utilization of the growing number of data systems. As the number of individual minicomputers increases, a system to interconnect them or substitute one for another becomes increasingly important (7). We are presently working on the transmission of entire files of data between computers by using existing hardware. This ability will provide additional flexibility to deal with the huge quantities of data that can be produced by modern analytical instrumentation.

References

(1) Chapman, J. R. (1978) *Computers in Mass Spectrometry*, Academic Press, London, UK
(2) Dusold, L. R., Dreifuss, P. A., & Sphon, J. A. (1978) 26th Annual Conference on Mass Spectrometry and Allied Topics, St. Louis, MO, May 28–June 2
(3) *4010 and 4010-1 Users Manual* (1976) Tektronix, Inc., Beaverton, OR
(4) Hunt, D. F., Stafford, G. C., Jr, Crow, F. W., & Russell, J. W. (1976) *Anal. Chem.* **48**, 2098–2104
(5) Heller, S. R., & Milne, G. W. A. (October 1980) *Online* 45–57
(6) Dusold, L. R., Dreifuss, P. A., Pohland, A. E., & Sphon, J. A. (1978) *Mycotoxins Mass Spectral Data Bank*, AOAC, Arlington, VA
(7) Dessy, R. E. (1977) *Anal. Chem.* **49**, 1100A–1108A

SIREN: Data Base Approach to Scientific Information Handling in the Bureau of Foods, Food and Drug Administration

GEORGE A. BRINDZA and ADRIANA McCANN
Food and Drug Administration, Technical Operations Staff, Washington, DC 20204

The Bureau of Foods has developed the Scientific Information Retrieval and Exchange Network (SIREN) to provide a modern method of records management and an automated cross-referenced subject index for accurate and comprehensive information retrieval. Authorized Bureau personnel may access SIREN's 200 000 references directly on distributed, interactive on-line terminals or indirectly through searches conducted by the SIREN Document Control Center.

The Bureau of Foods (BF) of the Food and Drug Administration (FDA) bases its regulatory decisions and actions regarding the safety and wholesomeness of the nation's food supply on the results of scientific research performed by its own staff and by other scientists. However, the rapid expansion of scientific and technical literature has made traditional methods of identifying, storing, and accessing this information obsolete. Although specialized filing systems adequately met the individual needs of BF scientists, they were virtually useless to other personnel and often led to a redundancy of data and a duplication of scientific effort. In response to the Agency's need for an integrated information system, an in-depth study was made to determine how BF could improve its method of handling scientific information. The internally generated and individually stored scientific information was viewed as an organizational resource, with costs controlled and benefits maximized, and a management strategy was formulated to establish an automated data base for scientific information storage and retrieval.

SIREN

Records Management

The Scientific Information Retrieval and Exchange Network (SIREN) was developed to provide a means for modern records management through strict control of the arrangement, microfilming, and accession of documents, and an automated, cross-referenced subject index. Although SIREN does not abstract, compare, or evaluate documents, it is valuable as an index which lists the precise locations of the information requested. As a central source of scientific information, SIREN makes the specialized, independently maintained research files easily available to all authorized BF personnel.

SIREN's approach to information processing, storage, and retrieval involves 4 major steps: document consolidation, microfilming, document control, and automated subject indexing. The first 3 steps relate to records management and are the foundation of SIREN; the fourth ensures consistency in entering and retrieving information through a controlled vocabulary.

Consolidation

Hard copies of files are gathered and purged of all duplication. Material is arranged, paginated, and bound in a standard manner. The new page numbers become the reference point for locating information on the microfilm or the hard copy and make it obvious when material is missing from the file. Although pages lost before consolidation can no longer be restored, any pages or whole files lost after consolidation can be re-created from the existing microfiche.

Recent consolidation of the Food Additives Information System (FAIS), a module of SIREN, included all significant internal files related to food additive research and regulation. More than 20 separate food additives files, containing about 3.5 million pages, had been originally maintained in triplicate, with one copy of each located in a different functional group in the Bureau. However, when a comprehensive review of all available safety information was needed for the food additives cyclic review program, retrieval of information from the triplicate files was virtually impossible. Some of the information had been lost, and the remaining material had to be reorganized and consolidated into a meaningful arrangement. After duplicate material was purged, FAIS files were reduced by 1.75 million pages and 600 linear feet of storage space, redundant files were eliminated, and a comprehensive file was established.

In a typical example, 3 copies of a petition file

Presented at the Symposium on Computers in the Laboratory, 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

0004-5756/82/6505-1268-03$01.00

were found in separate BF areas. The most complete copy contained 1000 pages (80% of the original); the others each had 700 and 800 pages. After duplicate material was purged, pages containing additional information were taken from the smaller files and added to the most complete file, bringing it to 1200 pages, almost 100% of its original complement. The 1500 pages of the 2 small files were then eliminated. Thus the original 2500 pages were reduced to 1200, the stored information in the most complete file was increased by about 20%, and the total number of pages was reduced by about 50%.

Microfilming

All documents in the SIREN system are photographed on microfilm, the hard copy is stored in its appropriate functional area, and 6 copies of the microfiche are distributed to various BF areas. The master microfiche copy, which serves as "backup" in the unlikely event that the original hard copy would be totally or partially destroyed, is stored separately in a remotely located vault.

Document Control

Document control ensures central storage of files, updating of information according to specific guidelines, and limited access to files. A main document control center, which supports all modules of SIREN, is available to the entire Bureau, and satellite centers are located in the appropriate BF branches.

Subject Indexing

Indexing terms used in SIREN are controlled by a thesaurus, which lists all terms or codes used for indexing and retrieving information from a particular file. The thesaurus designates preferred terms, the relationships between terms, and a hierarchical pattern, if appropriate. Paired relationships known as *broader* and *narrower* terms (BT and NT) express different levels of generality (e.g., BT plastic and NT polyethylene). Two preferred entries may simply be *related* terms (RT); if one is indexed, the other should also be considered (e.g., data processing, RT computers). SIREN uses codes to represent index terms (e.g., CAS registry numbers replace chemical names).

An alphabetic list of chemical names, a glossary of name sources, and a tree structure assembled from logical combinations of broader and narrower terms can be displayed and used for searching. Individual records are flagged and printed in subgroups or microthesauri, of which there are 18 in SIREN. These subsets include petitions, monographs, types of chemicals, routes of administration, dosage forms, subjects of experimental work, and chemistry and toxicology topics. When multiple codes are selected, a larger group such as the entire chemical authority list, is printed. In SIREN, this list amounts to more than one-half the thesaurus.

Chemical Names

The total number of thesaurus entries is 65 000, with about 37 000 preferred terms. Chemical names with their CAS registry numbers account for nearly 19 000. Drug names once accounted for the largest group, followed by cosmetic ingredient names and food additives. However, the general chemical dictionary, which lists possible degradation products, contaminants, metabolites, or manufacturing intermediates of petitioned food additives, is now the fastest growing section of the chemical file.

Preferred names for chemical substances include common or established names assigned to direct or indirect additives, cosmetics, and drugs under legislative and regulatory directives. Common or trivial names are assigned to natural products, e.g., resins. Natural substances, such as algae, rice bran oil, and *Salmonella* are all treated as chemical substances; however, they have no CAS registry numbers. Some general or collective names, for which the exact chemical designation may have been lost or may never have existed, are not always precise, as in C 13–16 isoparaffins, carrageenan, and salts. Others, like fatty acid esters and fatty acid salts, are actually defined in the Code of Federal Regulations.

As a chemical name authority list, the thesaurus supplies the following: preferred chemical name; current CAS registry number; internal number when there is no CAS number; alternative names; and designator or flag, indicating the FDA program interest of the compound, such as direct food additive, indirect additive, flavor.

Vocabulary Control

When an index record is added to the SIREN file, the computer checks the code against the thesaurus vocabulary. When records are retrieved, the codes are again compared with the thesaurus table, translated into chemical names or index terms, and printed on the index record.

Indexing Levels

SIREN indexing is provided at 2 different levels. Level 1 data elements describe the doc-

ument as a whole (e.g., the name of a submitter of a petition, the petitioned chemical, or another chemical with a specified relationship to the previous one). Level 2 data elements describe specific toxicology information, chemistry or manufacturing information, and memoranda within petitions and other documents. A detailed indexing guide ensures consistent indexing.

Discussion

SIREN operates at the Parklawn Computer Center (PCC) in Rockville, MD, on an IBM 3033 central processor. The data base is managed by Model 204, which is supplied by Computer Corporation of America. In the past, hard copies of BF documents, previously stored in more than 25 individually maintained files, could not be cross-referenced; now, however, SIREN provides concurrent on-line and batch access to approximately 300 000 references simultaneously to many users throughout FDA. Through 1986, more than 65 000 references are scheduled to be added yearly to the system. Because of SIREN's success, additional Bureau files are being considered for placement in the network.

Because of the need to protect the confidentiality of information stored in SIREN, only those authorized within FDA are permitted direct access to the system. Persons outside FDA may use SIREN indirectly through proper FDA channels. Access privileges must be determined on a case-by-case basis. For further information on the possible use of SIREN, please contact: Hamilton Parran, Deputy Director, Division of Food and Color Additives, 200 C St, SW (HFF-330), Washington, DC 20204. (202) 472-5676.

Data System and Software for a Fourier Transform Infrared Spectrometer

JOHN W. PHILBRICK and WAYNE F. HERR[1]
IBM Instruments, Inc., Danbury, CT 06810

The computer described has as its primary purpose the control of an FTIR spectrometer. This task requires enough power to handle the rather high peak load of this instrument. Software has been written to flexibly shift the system resources to meet this task, and to make these resources available to the spectroscopist for other jobs as required. Clearly these jobs include the routines of collecting, transforming, storing, and displaying the spectra of interest. The capabilities described go beyond that, to include the ability to handle an automatic sample changer, to vary the measurements made on samples within that sample changer as required, and to annotate the resulting plots with much of the routine bookkeeping data required in an analytical laboratory. This system has the ability to multitask – to perform up to 3 tasks at a time, or to pipeline – to automatically pass data from one region to another. Both of these provide enhanced productivity. The system supports high level language, for the user's own analyses. It has facilities for library searching, to help identify the unknown samples. Finally, it has text processing, to allow the user to prepare reports and papers in a modern way. In short, the line between an instrument computer and a laboratory computer has been blurred by the growth of the capabilities of the former.

The basic job of a laboratory computer is to make its user more productive by handling some or all of the data handling faced by a scientist in a laboratory (1). These include:

Bookkeeping—Keeping track of samples and data.

Instrument control—Setting key instrument parameters, monitoring instrument condition, and controlling operation.

Data acquisition—Acquiring raw data from a sample during analysis.

Data analysis—Converting raw data into a more meaningful form.

Data interpretation—Converting analyzed data into an answer.

Storage—Placing the data and the answer into an easily recovered file.

Report generation—Presenting the data in a fashion that helps the scientist's organization or science in general.

The computer(s) used by the scientist in his/her job should impose minimum requirements on the scientist, in terms of making the analyst learn new procedures or restricting the way the data can be stored, manipulated, or presented. As much as possible of the job should be handled in ways which are transparent to the user—file management and data movement for instance should be all but invisible.

Using this brief list, laboratory computers might be divided according to the tasks they handle. Instrumentation computers are those which handle data acquisition and perhaps data analysis and/or instrument control. General purpose laboratory computers might be seen as those which handle the bookkeeping, storage, and data interpretation part of the job. The report generation part is largely omitted in laboratory computing, yet as avid users of text processing, we feel that such omission is a serious oversight.

In this paper, we will consider the distinction between general purpose laboratory computers and specialized instrumentation computers, and describe how the growing power of instrumentation computers in particular is leading them to reach into the domain previously handled by larger machines. We will focus on the data system in a Fourier transform infrared spectrometer (FTIR) as an example of an instrumentation computer.

Laboratory Computers

Computers first appeared in laboratories either to perform calculations, or to manage data bases. The latter is particularly important in clinical laboratories (2), and has not been as widely developed in analytical laboratories (3). Later, these computers began to actually connect to instruments. This function fell away as dedicated minicomputers appeared under the covers in instruments. It has gradually been restored as mid-sized lab computers gained the ability to handle more than one problem at a time. Currently, the line between the instrumentation computer and the laboratory computer may blur,

Presented at the Symposium on Computers in the Laboratory, 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

[1] IBM Corp., Poughkeepsie, NY 12602.

0004-5756/82/6505-1271-08$01.00

with the dividing line being determined by where the data are actually digitized.

More commonly, the larger laboratory computers are supporting multiple users, each with the ability to access and manipulate his own set of data, and yet with each having the ability to contribute or access data in a larger data base. These larger machines may be used for library searching, for large theoretical calculations, for archival storage of data, and for text processing of reports. We are not advocating that scientists become typists, but if the keying process is part of the creative process, why not have the author produce legible output using the extensive power of word processing? Watching the number of people who are using home computers to write letters, papers, and books suggests that this capability is going to be in more and more demand on laboratory systems.

The data base management capability is another under-utilized feature. Why have dusty notebooks full of data, or rolls of spectra stuffed away in boxes, when a computer can store tremendous volumes of data for almost instant access? All that is really needed is a cataloguing system, and the discipline to use it. This should include the ability to link textual files, containing material describing the sample's history and the details of the analysis, with the numerical data resulting from the analysis.

Finally, networking is important. This paper was written with little direct contact between the 2 authors. One of us (JWP) keyed his contributions on a terminal located in Connecticut and connected to a computer in Yorktown Heights, NY. The other (WFH) keyed his data on a terminal in one building in a large complex, connected to a computer located close to a mile away in another building, both in Poughkeepsie, NY. The data were transmitted from one machine to another through a network of perhaps 5 other computers. The reader may judge whether or not the quality of the work suffered; certainly it was a very convenient way to work.

Instrumentation Computers

An instrumentation computer could do as little as collect and analyze the data (however large this job may be), or it could provide control of the basic instrument. For instance, a chromatographic integrator may simply acquire and perform a preliminary analysis on data which would otherwise be presented on a strip chart recorder. A more sophisticated chromatographic controller might also control the mixture of the carrier gasses or liquids being used, or might control column temperature. In either or both of these cases, these variations could be preset and changed in a time-dependent fashion, allowing more sophisticated analyses to be run in a shorter time. These control functions could be performed manually, depending on the times involved, or could be performed with analog instrumentation if needed.

Some instruments require the computer for data analysis. NMR spectrometers may require signal averaging to obtain reasonable signal to noise values. Pulsed or Fourier transform NMR spectrometers require the computer to make sense of the data—interpretation of the magnetization decay is difficult in the frequency domain, but may be done in a more straightforward fashion in the time domain.

The computer in a Fourier transform infrared spectrometer was originally placed there to convert the data into a comprehensible form (4). The retardation data have direct meaning in only a very few cases—thin film measurement is one possibility. Even there, more information can be extracted from the interferogram, or the data may be more accurately interpreted if they are transformed into the more familiar frequency domain.

Once the computer was included in the instrument, additional functions were added. Signal averaging was present from the start. Control of a plotter was a necessity—we are used to seeing spectral information displayed graphically. Some additional capabilities remained within the domain of the traditional instrumentation computer, like controlling various features of the instrument, e.g., sample changers, beam splitters, sources, and detectors. Others moved more into the realm of the general laboratory computer. File management, as larger disks and as tape drives were added, was necessary. Additional analysis, moving towards interpretation, was another. This can take the form of spectral comparison, either simple subtraction of one spectrum from another to eliminate an unwanted component, or the more complex comparison of the spectrum of an unknown with those stored in a library. Greater analysis flexibility was added by including the ability to write software in high level languages, rather than the assembly code used at the start. Thus, the user could tailor at least the analysis and interpretation of his data to match his own needs.

FTIR Data System Tasks

The data system for an FTIR must provide for rapid data collection; Fourier transform support;

mass storage; flexible control system; high level language; hard copy output (graphics and text); productivity/automation capabilities. We will discuss the first 3 of these here; the others will be amplified below.

The data collection job of an FTIR computer is really quite impressive. At high mirror velocities, the analog to digital converter (ADC) must sample at speeds up to 100 KHz. With a 16 bit ADC, this is a bit rate of 1.6 Mb/s. While the actual duty cycle may be about 50% (data may be acquired only while the mirror is moving in one direction), this still requires rapid movement of the data. The data are acquired into memory or to disk, depending on the resolution and hence the number of data points being acquired. The new data are then correlated with the previously acquired data, and may be rejected if they appear to have been shifted with respect to the earlier data, perhaps due to a mechanical jolt to the instrument or due to an electronic noise spike. If accepted, the new data are then added to the data already stored in memory or on disk. All of this must be done on an interrupt-driven fashion, because the computer must be ready to repeat the digitization, acquisition, signal averaging, and storage cycle as soon as the mirror returns to its start position.

The transformation itself is a complex computation, which has been massively studied and documented (5). Briefly, it can be broken down into 3 separate computations—phase correction, apodization, and transformation. The first 2 correct for the difference between the theoretical infinite, complex mathematical domain of the Fourier transformation, and the real, finite nature of the data actually being acquired. The transformation itself involves the use of an array of sine values, which are most conveniently looked up from the values stored in read-only memory in the computer. The density of these tables (the number of values stored there) is important in ensuring the accuracy of the final calculation. These 3 operations involve multiplications and additions of each point in the initial interferogram, or a number of operations of the order of 8000 for a 2 wavenumber, mid-IR spectrum. Clearly, all of these calculations must be performed rapidly for the instrument to be sufficiently responsive to allow the chemist to do his job without being delayed by instrumental factors.

There never seems to be enough memory, either on-line or in mass storage. While a 2-wavenumber mid-IR spectrum may use only 8K data words, higher resolution spectra require proportionately more room: 0.5 wavenumber spectra require 32K data words; 0.05 wavenumber spectra require 320K words. Clearly, high resolution demands either large memory or the ability to swap data in and out of memory quickly.

A reasonable library of spectra can also bulk up quite quickly: 5000 2 wavenumber spectra require 40,000 data points. Since disk and tape sizes are measured in 8 bit bytes, and FTIR computers work in 16, 20, or 24 bit words, that 5000 spectra library can require up to 120 KB on a disk or tape.

The volume of data which can be generated by techniques as gas chromatography/infrared spectrometry (GC/IR) or time-resolved spectroscopy (TRS) is even more massive. In GC/IR, the FTIR is used as the detector, taking advantage of the spectrometer's ability to acquire several spectra per second. A 4 wavenumber spectra yields 4K data points per spectra. Even co-adding spectra to yield one per second, a run of slightly over an hour can accumulate 16M data points, or 48MB of data. Clearly, either a large disk or some technique for selectively storing data is required.

The resources the computer can bring to bear on these and its other jobs are shown in Figure 1.

Figure 1 shows the computer and its associated peripheral devices, with the instrument itself shown only as a collection of input/output devices. Note that the software is sufficiently powerful to allow several things to happen at once. There is a supervisory system, which interacts with the various peripherals including the terminals, the disk, the diskette drive, the instrument interface, and the plotting devices. In the ASPECT computer, used on the IBM IR/90 and IR/80 series of instruments, the supervisor is known as ADAKOS. Given enough memory, ADAKOS can run 2 memory partitions. Typically, one partition would be used to operate the instrument, and a second might be used for program creation or off-line data analysis, using a second terminal. The instrument operating system, ATS on the ASPECT, may in turn be divided into 3 regions as shown in this figure, for additional operating efficiency. All of this will be illustrated below.

Computer Usage

Perhaps an easy way of illustrating the demands made on a computer system, and the power such a system provides its user, can be given by following a typical working session for

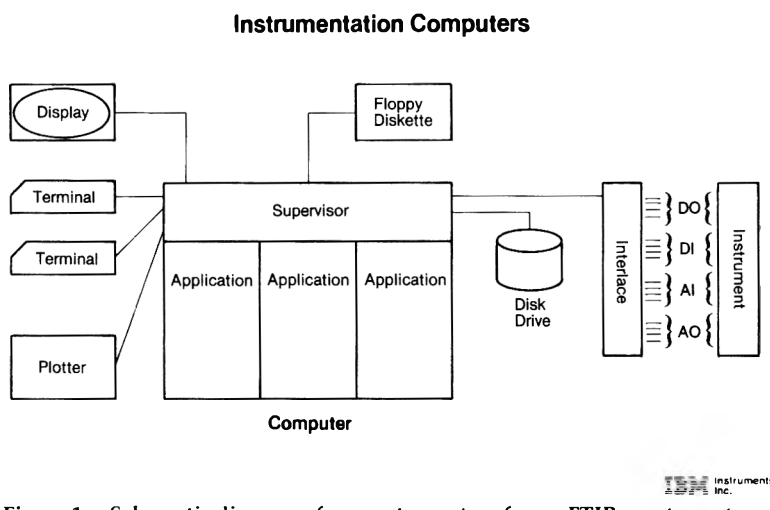


Figure 1. Schematic diagram of computer system for an FTIR spectrometer.

a spectroscopist. In this example, many of the details will be drawn from the ASPECT data system using ADAKOS and ATS. In addition, some comments will be made about additional facilities which might be desirable.

Instrument Check and Verification

Instrument Check

At the start of a session with an instrument, the chemist needs to check and verify the instrument performance, then set it up to do the job he needs. Some systems require an Initial Program Load (IPL) from some external medium. Older instruments use paper tape; more recent systems will load from a floppy diskette, ROM, or will have the programs and data stored on a disk.

Traditionally, spectroscopists check an IR spectrophotometer by running the 100% line, the plot of the instrument's response to a perfectly transparent or null sample. This is as valid on an FTIR as on a dispersive instrument. The 100% line check uses almost all of the system: the keyboard, the data system, the optics, and the display. It really uses a complex array of commands and parameters, but can be invoked with a single command, as shown below. The meaning and function of the individual commands is beyond the scope of this paper; the important point is that one command, defined by the user, can recall 8 primitive commands, and that one of those primitive commands in turn can recall some 39 instrument parameters.

The ATS program allows the user to define, store, and recall complex operations very easily. User-defined commands and parameter sets may be specified in the normal sequence of operations. The sequence of commands is entered, and the parameters are set. If the resulting operation meets the user's objectives, the command sequence may be identified and saved, using one command which saves the information under a name entered in response to a prompt.

HPL: Commands for 100% line check

Invoked Command	*Meaning*	*Parameters Used*
RUP UDP = PARAMHPL	restore user parameters	39
SBS	single beam calibrate, sample	
SBR	single beam calibrate, reference	
CLR	collect reference	(19)
CLS	collect sample	(2)
CPR	compute reference	(10)
CPS	compute sample	(2)
PLT POP = DSC	plot	(8)

Polymer Film Verification Run

The next step is to check the instrument on a test sample. Traditionally, a polymer film has been used. This run uses many of the same commands and parameters used for the 100% line, and could be set up by simply changing some of the parameters stored in a data file named PARMHPL. Because these data may be retained as part of an ongoing record of instrument performance, the plot should be sent initially to the display scope for verification, and

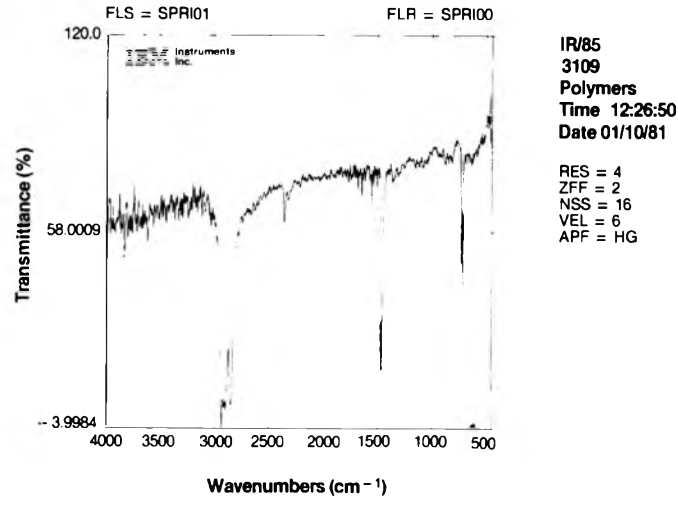


Figure 2. Uncorrected polymer film spectrum.

then to the digital plotter for permanent record. The commands to do this are shown below.

VPF: Commands for verification run on polymer film

Invoked Command	*Meaning*	*Parameters Used*
RUP UDP = PARMVPS	restore user parameters	39
SBS	single beam calibrate, sample	
SBR	single beam calibrate, reference	
CLD	collect dual	(21)
CPD	compute dual	(12)
PLT	plot to scope	(8)
RUP UPD = PARMVPP	restore user parameters	2
PLT	plot to plotter	(8)

Again, one user-defined command invokes a series of actions, including invoking more complex commands and recalling preset parameters. The parameters recalled from PARMVPP change the plotter output from the scope to the plotter, and add certain labeling to make the plot a permanent record. Three 16 character variables may be specified by the user, included in the data file itself, and listed on the plot. The results are shown in Figures 2 and 3. The computer was programmed to automatically label the plot, and to include both the date and time of the run. It also included an independent sequence check: The number "6109" on both plots shows the total number of scans made by the instrument since the computer was powered up. This running scan number is of great assistance in reordering a set of plots after having been removed from the plotter and shuffled on the chemist's desk. The information on number of scans, resolution, and zerofilling, among other acquisition parameters, is also stored along with the actual data: The system is operating automatically like a laboratory notebook.

The difference between Figures 2 and 3 is instructive. Figure 2 is an uncorrected plot of the polymer film transmission, with sloping baseline due to the light scattering within the film. Figure 3 has had the slope corrected, using some built-in software routines. It has also had the various peaks picked out, stored in a separate file, and labeled on the plot.

While all of this annotation is in one sense trivial (person can easily make a couple of notes on the paper while the plotter is running) it is an example of the way the computer can take over the trivia, ensure that it is handled, and free the analyst to do the creative work that is in fact that person's real job.

Note that the particular command sequence shown above included no commands for file movement. The system automatically handles this for the user. An additional capability is shown on the top of Fig. 2, where FLR and FLS call out the names under which the reference and sample data are stored. Up to 8 alphanumerics may be used for these names, for easy recognition by the chemist later. Note that the system can

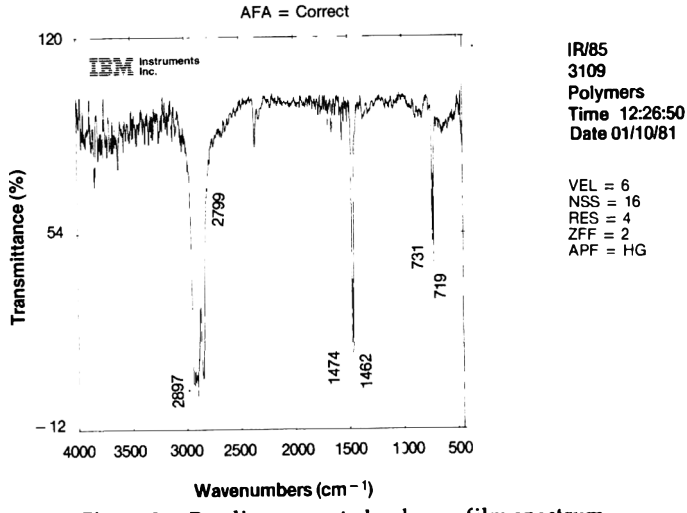


Figure 3. Baseline-corrected polymer film spectrum.

also increment the file names, as shown here, if sequential samples are to be measured.

Multitasking and Pipelining

Multitasking

Figure 1 indicates that the instrument computer can perform multitasking. In essence, this means that the user can assign it up to 3 separate jobs at once. The software will then go ahead and work on each of these tasks, sharing the resources and improving productivity by working on each task as the required resources become available. For instance, while one region is collecting, it needs to have immediate access to the disk for data storage, but it may only need this access 50% of the time. While the interferometer mirror is moving back to its start position, the disk is largely available for another job. Multitasking takes advantage of these time-varying requirements on system resources to apparently accomplish more than one job at a time.

Pipelining

A more sophisticated variation on multitasking is called pipelining. This mode requires communication between the various tasks, in the form of (a) data movement from one region to another, and (b) setting and reading of flags such that one region can notify another about the availability or the consumption of the data. A typical operation using pipelining is shown in Figure 4. This figure assumes that a series of samples are available to be measured. The actual data are collected in region 1. As soon as the data are collected on a given sample, region 2 is notified via a system of flags. This region can then transform and analyze the data while region 1 is collecting data on another sample. If the user assigns subtasks of about equal time duration to each region, time savings approaching 50% can be achieved.

Handling Multiple Samples

Typically, more than one sample will be handled by a spectroscopist at a time. Indeed, he may have a whole rack of them. Some of the FTIR instruments now have automatic sample changers with software selection of the specific sample to be measured. It is straightforward to use identical measurements on all of those sam-

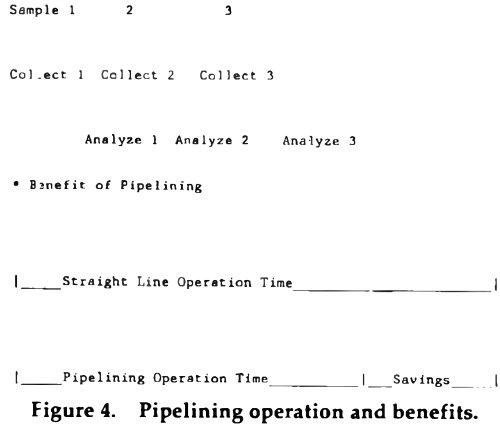


Figure 4. Pipelining operation and benefits.

ples. Of more value is the ability to select the measurement conditions appropriate for each individual sample. Dense samples may get more scans to improve the signal-to-noise ratio; relatively transparent samples may be measured more quickly. It may be known in advance that some samples will require peak picking and labeling; some chemists may want to see their data presented in one format or at one resolution; others with other variations. Software which allows this flexibility offers the possibility of unattended measurements and hence a highly productive way of running. ATS has the capability of not only providing individualized measurements, but also of providing individually annotated plots. Thus at completion of a run of a series of samples, there is an array of plots sitting on the plotter, each labeled and ready to be handled back to the requester. Furthermore, since the ASPECT can intermix files of different sizes automatically, each specimen may be measured at an appropriate resolution.

Library Searching

A fundamental job of the analytical chemist is the identification of the specimens presented to him. Infrared spectroscopy provides a powerful tool for this purpose, because it looks at the very electronic interactions and bondings which cause a compound to exist and behave as it does. This information appears in the familiar form of absorption bands of various strengths and frequencies in an infrared spectrum. Clearly, comparison of the unknown spectrum with that of spectra from various known compounds can aid in identification. Alternatively, knowing that various functional groups typically create absorption bands at characteristic frequencies may enable the spectroscopist to do some detective work to identify the unknown. In either case, the spectroscopist must ultimately confirm the identification based on a visual comparison of the unknown and candidate spectra.

This process can be accelerated if the computer can do some of the winnowing for the spectroscopist. There are many ways of doing this, trading off the speed of the search with accuracy of the ultimate identification. Using more of the points in the various spectra may yield a closer match than using fewer, but it may also take longer. One long accepted approach is the coding scheme used in the Sadtler Spec-finder® in which a reduced form of the spectrum is created and searched against similarly reduced data in the library. This reduction allows storage of all 60 000 spectra to be on line at once to be searched within 10–20 s. The candidates may then be confirmed by comparison with published and catalogued data, or with data automatically retrieved using a microfiche system. ATS software also allows the user to add spectra from samples of his own choosing to the library, to meet particular requirements of his own organization. Other searching techniques are being studied in the industry, including searching on the full spectrum, or searching on a portion of the interferogram.

High Level Languages

While instrument manufacturers use consultants and their own staff of experienced spectroscopists to define and write the software for their instruments, many spectroscopists have their own particular requirements and want the ability to tailor the data analysis and instrument operation accordingly. The instrument manufacturer frequently writes his software in the assembly language peculiar to his own computer, for speed of execution. The spectroscopist would prefer to work in something more familiar and, preferably, something which already has a library of useful programs. PASCAL and FORTRAN meet both of these requirements.

PASCAL is a structured language, which is easy to learn and currently quite popular on campuses and on home computers. Programs written in this language are easy to read, and hence easy to modify and to maintain.

FORTRAN has the advantage that many practicing scientists learned it during their careers. Further, a considerable library of programs has been created in this language. Unfortunately, there are a number of dialects of FORTRAN; hence not all of those programs are immediately available to the user of a given version.

Text Processing

A rather neglected area of computer usage in laboratories is text processing. This is probably one which is going to have rapidly growing importance. It is intriguing to note the number of hobbyist computers appearing on the desks of faculty on college campuses. These are not being used for computation, but instead are being used to write books or papers.

That capability is provided as part of our system, using programs initially written by James Cooper of Bruker Instruments, Inc. They were initially written to provide his scientists and programmers an easy way to write the manuals and document the programs on the instruments

being offered by that firm. This facility offers the user the ability to format and justify text; operate on files created with the normal system editor; create indices; automatically number pages and chapters; create subscripts and superscripts, depending on the printer; conditionally include or exclude text; indent, center, underline, and print boldface text.

Acknowledgments

The authors are grateful to J. Grunholz and H. Somberg of Bruker Analytische Messtechnik, GMBH, and to Eric Rosencrans of IBM Instruments, Inc., for their creative work on much of this software, and to J. C. Casper and A. J. Rein for their work in helping to define this system.

References

(1) Buchanan, E. B. (1979) Criteria for Selecting a Lab Microcomputer System, ACS Symposium on Choosing a Laboratory Computer System

(2) Sprayberry, M. N., et al. (1976) *Am. J. Med. Technol.* 6, 201–208

(3) Frazer, J. W., & Barton, G. W. Jr (1975) A Feasibility Study and Functional Design for the Computerized Automation of the Central Regional Laboratory EPI Region V, Chicago, ASTM STP 578, American Society for Testing and Materials, pp. 152–255

(4) Griffiths, P. R. (1975) *Chemical Infrared Fourier Transform Spectroscopy*, John Wiley and Sons, New York, NY

(5) Cooper, J. W. (1978) "Data Handling in Fourier Transform Spectroscopy," *Transform Techniques in Chemistry*, P. R. Griffiths (Ed.), Plenum Press, New York, NY

A Distributed Processing Laboratory Data System

THOMAS J. BIRKEL[1] and LAURENCE R. DUSOLD
Food and Drug Administration, Division of Chemistry and Physics, Washington, DC 20204

Distributed data processing has been accomplished by a computer system in which laboratory instrument data are collected on a PEAK-11 system for preliminary processing and generation of initial reports. When further processing is required, or when archival storage of raw or processed data is desired, data are transferred over telephone lines to an IBM 3033; an IBM 7406 Device Coupler is used to handle protocol conversion and "handshaking." User-written programs in APL.SV on the IBM machine and in Assembly Language on the PEAK-11 system effect the transfer of bidirectional data. The distributed processing approach allows efficient use of expensive peripherals while maintaining short response times.

A PEAK-11® Laboratory Data System and an IBM 3033 remote mainframe have been connected for use in distributed processing of data in the Food and Drug Administration (FDA). The total system is being used to collect data from 16 instruments physically located on 2 floors of FDA's Bureau of Foods laboratory facility. The types of data being collected concern food packaging, various types of food additives, electrochemistry, and radiochemical detection.

The laboratory data system is a PDP-11/34A that utilizes PEAK-11 applications programs (Figure 1). These are divided into 2 major groups: (1) data acquisition and processing, and (2) report generation and parameter set-up and change.

The foreground job runs in real time under the RT-11 Version 3B Foreground/Background operating system. It is an assembly language routine which schedules collection of analog data from up to 16 channels "simultaneously" at speeds of 20 Hz per channel. The same routine processes the raw data for peaks, and stores raw data, peak data, or both on a disk.

The background job, in our version of RT-11, runs during the CPU idle time. In the PEAK-11 system, MU-BASIC, a multi-user version of BASIC, runs as the background task. "Canned" programs running under MU-BASIC handle all communication between the users and the foreground task, as well as reporting of processed peak data.

This configuration offers a great deal of flexibility, because several users can be changing analysis parameters, getting reports, or programming in BASIC concurrent with data collection and processing of up to 16 analog data signals. It does, however, have one major disadvantage over a true multitasking operating system (Figure 2). Under this monitor, only 2 jobs or tasks are allowed at any one time, and both of the Peak-11 tasks must be running for the user to get reports, change analysis or acquisition parameters, or program in BASIC while data are acquired.

The other computer facility available to us (Figure 3) was the Parklawn Computer Center (PCC), located approximately 30 miles away, which provides services to the Food and Drug Administration as well as several other federal agencies.

As Figure 3 illustrates, the hardware available at PCC included (but was not limited to) dual CPUs with parallel access to disks, magnetic tape drives, mass store units, etc. These large devices, although desirable, are uneconomical for a small laboratory system.

In addition to the hardware, several software advantages were available at PCC. FORTRAN, BASIC, COBOL, ALGOL, PL1, RPG, SNOBAL, and APL were among the language choices. We chose to use APL for most of our programming at PCC because of its ease of use and its ability to handle variable length strings easily.

With these systems already configured as described, our most serious problem in establishing a distributed processing system was to find a suitable method for information interchange between the CPUs.

Digital Equipment Corporation (DEC) sold a combination hardware/software package for communication between a PDP-11 series CPU and an IBM 370 mainframe. Their 2780 Remote Job Entry Subsystem transfers entire files between processors. However, the particular version suitable for use with our operating system could require 90 of each 100 milliseconds of CPU time to handle time-dependent protocol

[1] Present address: General Foods Corp., White Plains, NY 10625.

Presented at the Symposium on Computers in the Laboratory, 95th Annual Meeting of the AOAC, Oct. 19-22, 1982, at Washington, DC.

0004-5756/82/6505-1279-04$01.00

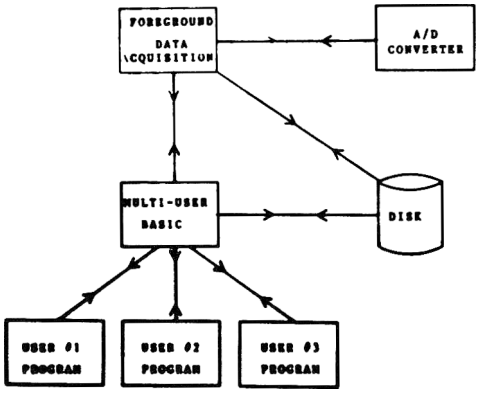


Figure 1. Laboratory data system.

requirements. The PEAK-11 foreground job could require, under certain circumstances, as much as 50 of each 100 milliseconds of CPU time. Thus we would be required to suspend data acquisition to ensure data transfer. This was not an acceptable solution in our laboratory where samples frequently were automatically injected around the clock.

We were therefore limited to considering approaches that would allow transfer while both PEAK-11 tasks were running on the laboratory computer. We considered and discarded notions of physical transfer of disks or magnetic tape between sites because we desired essentially "on-line" turn around times for transfer. The approach we finally adopted involved commercially available hardware and user-written software to transfer data, one word at a time, between CPUs.

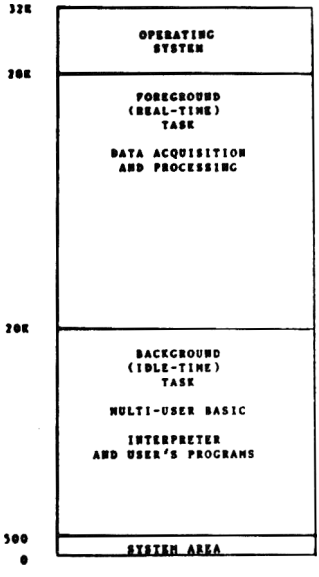


Figure 2. True multitasking operating system.

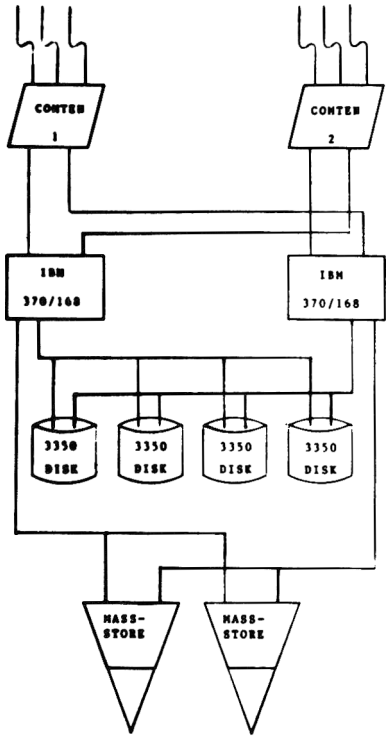


Figure 3. Computer system facility.

The hardware included a 16 bit parallel interface (a DR-11K from DEC) which plugs directly into the PDP-11/34A Unibus® (Figure 4), and an IBM 7406 Device Coupler connected into the

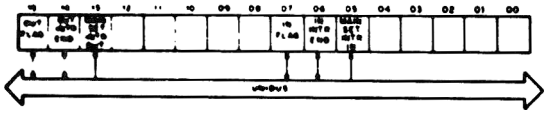


Figure 4a. Status register data flow (↑ write/↓ read).

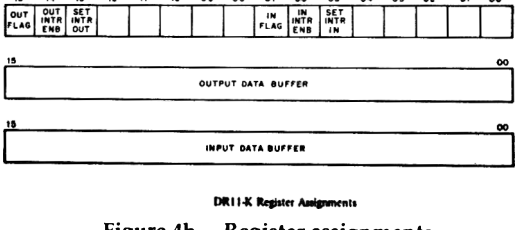


Figure 4b. Register assignments.

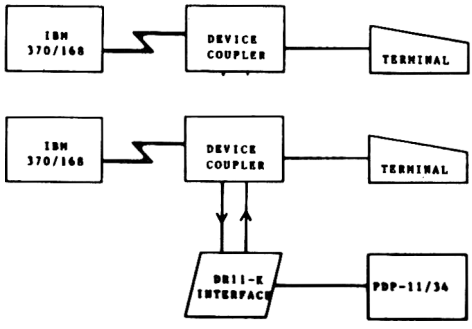


Figure 5. Device coupler operation.

telephone line between the IBM 370 host and a remote terminal (Figure 5). The 7406 Device Coupler was equipped with Digital Input (DIM) and Digital Output modules (DOM) as well as the standard System Communications Module (SCM).

Because the operation of the Device Coupler is somewhat unusual, we will briefly mention only a few of the salient points. Normally the device coupler merely passes data between the host CPU and a terminal, as shown in Figure 5. However, when a special character sequence appears on the communication line, the SCM "traps" the following character string and interprets it as a command. Depending on the nature of the command, the SCR activates the appropriate module to take action (see Figure 5). The following is the general format of a Device Coupler command string:

,SZcmad—d,F

where:

,S	is the character sequence which activates the System Communication Module (SCM)
Z	is a required command flag
c	is the command W to write to a module R to read from a module
m	is a command modifier
d—d	is data sent to the addressed module
,F	is the character sequence which disables the SCM

The DR-11K module consists of independent buffered input and output registers and a status register, each of which is individually addressable on the Unibus.

In contrast to the hardware, which was all commercially available, all software for our system was written by chemists in the laboratory. The software consisted of 4 major sections:

1. APL.SV programs at PCC to activate and control the Device Coupler and decode incoming data;
2. JCL instructions to handle transfer between APL.SV and other PCC partitions;
3. MU-BASIC programs on the PEAK-11 system to access data files and handle macro level protocols;
4. Assembly Language routines linked to MU-BASIC to control the DR-11K in a manner similar to a device handler routine.

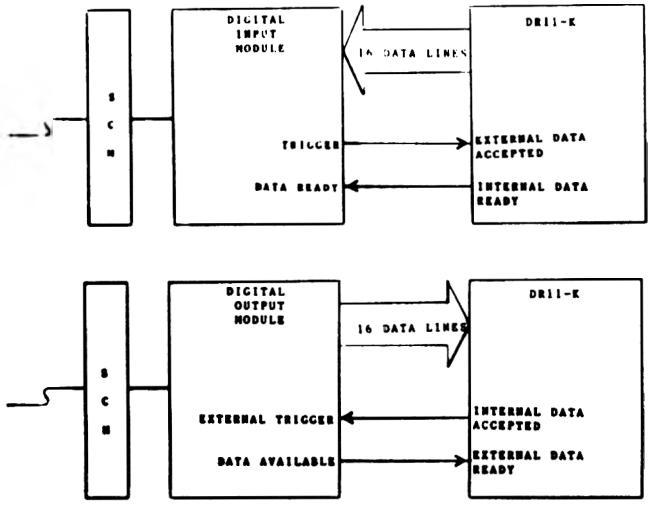


Figure 6. Data transfer operation.

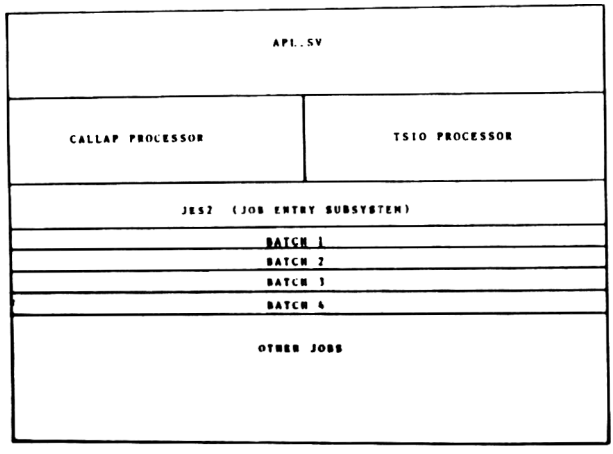


Figure 7. Shared variable facility.

The actual transfer involves 2 distinct levels of protocols. From the program, or macro level, the user running an MU-BASIC program calls a subroutine, DRCO (A,B) for output or DRCI (A,B) for input, with 2 arguments. A is the value or variable name to be output or input. B is a control flag. The subroutine called handles the micro or word-level transfer on the PDP-11. Similarly an APL.SV program sends a character string to the Device Coupler, activating it for a word transfer.

The following example of data transfer from the PDP-11 to the IBM 370/168 may help to clarify the situation. The MU-BASIC program calls DRCO with the value to be output. The assembly language routine DRCO loops either for a given time or until the input flag is set in the DR-11K status register. Meanwhile, the APL.SV program at PCC sends a character string to the Device Coupler, activating the DIM to signal the DR-11K, indicating that the DIM is ready to accept data. The trigger pulse from the DIM sets the input flag of the DR-11K status register. The data word is loaded into the output register, and the DR-11K pulses the DIM over the control line (Internal Data Ready) that valid data are available (see Figure 6).

The routine DRCO clears the status word flag, sets the control flag B (to 1 for successful transfer, to 0 for a time-out), and returns to the calling BASIC program. Meanwhile, the DIM inputs the data, transfers it through the SCM to the calling APL.SV program, and is deactivated.

Transfer from PCC to the PDP-11 system occurs in a completely analogous manner, with the MU-BASIC program using the routine DRCI and the APL.SV program activating the DOM for transfer (see Figure 6).

At the macro or program level, certain conventions are followed. The first 2 words transferred indicate the total number of items to be transferred. The programs at each location can thus retain the flexibility to transfer files of variable length without reprogramming.

From the APL.SV workspace through the shared-variable facility using the JCL routines, we have access via the TSIO and JES2 processors (see Figure 7) to essentially all the resources of the large mainframe system. This provides several direct and indirect benefits. For example, each Mass Store unit has the capacity to store over 30 *million* chromatograms of 60 min duration with data collected at 20 points/s. The direct benefits include reducing laboratory space dedicated to data storage. From this same example, however, we derived several indirect benefits. The Mass Store unit automatically makes a duplicate copy of each record as it is being stored, thus assuring backup of critical data. In addition, the security arrangements already in place at PCC eliminated all but very modest expenditures at our site to limit access to data.

In summary, we have presented the concepts and some of the details of the approach we took in establishing a communication link between a PEAK-11 Laboratory Data System and an IBM 370/168 mainframe for distributed processing of laboratory data. Our approach allowed us access to resources which could not be economically supported in the laboratory, and it resulted in cost savings for several overhead activities.

TECHNICAL COMMUNICATIONS

Design of an All-Metal Syringe Heating Mantle

B. DENIS PAGE and CLAUDETTE F. CHARBONNEAU
Health and Welfare Canada, Food Research Division, Health Protection Branch, Ottawa, Ontario, Canada K1A 0L2

The design and fabrication of a simple, robust, all-metal heating mantle, readily adaptable to different sizes of gas-tight (or other) syringes, is described.

Headspace gas chromatography (HSGC) has become an increasingly powerful tool for the determination of volatile compounds in liquid or solid matrices. In recent years, several books and review articles have been published (1-3) on the quantitative aspects of this topic. In trace analysis by HSGC, the volatile components from the matrix can be concentrated by using a purge and trap technique before chromatography. Alternatively, the matrix to be analyzed may be sealed in a closed system, and when an equilibrium of the volatiles between the gas and the matrix is reached, an aliquot of the headspace gas can be removed and analyzed by gas chromatography. To increase the concentration of volatile components in the headspace, the equilibrium temperature of the headspace sample vial may be increased. With automated headspace sampling equipment, thermostat-controlled heating of the sample vial and the injection mechanism is readily achieved; however, this equipment is expensive and involves either an injection system or a gas chromatograph dedicated to HSGC. Manual headspace injection, however, can be readily carried out on conventional gas chromatographs and requires only a heated syringe to approximate the automated procedures. To obtain the necessary rapid and reproducible heating, a syringe mantle, in which only the needle and hub are not directly heated, can be used.

Ideally, the temperature of the syringe should be reproducibly adjusted, by using a thermostat, to the same temperature as the sample or to a higher temperature. This avoids problems arising from the condensation of hot sample gases onto the cold syringe barrel or other syringe parts. If such condensation occurs, the small quantities of condensate, containing the volatiles, possibly in a different proportion to the noncondensed gas, may be injected into the gas chromatograph. Thus, acceptable repeatability may be difficult to achieve. Even the use of an internal standard may not compensate for this problem. Condensation in the syringe could be minimized by heating the syringe in an oven; however, such heating would not be rapid, and reproducible syringe temperatures, except after prolonged heating, would be difficult to attain. The resulting varying syringe temperatures could adversely affect repeatability. All-glass heating mantles for Hamilton gas-tight syringes, which permit uniform reproducible heating to prevent condensation problems, have long been available (Hamilton Co., Reno, NV). These glass mantles, however, are fragile and generally not adaptable to syringes of other manufacturers.

This note describes a readily fabricated, inexpensive, robust metal syringe mantle. The design of this mantle can be easily modified to fit gas-tight or conventional syringes of different capacities and manufacturers. In addition to headspace analysis, applications requiring a heated or cooled syringe are readily accommodated.

Fabrication

To a piece of 1.5 or 1.25 in. id copper pipe (part A), solder 2 circular end plates (part B) of 0.0625 in. thick copper sheet as shown in Figure 1. Drill two 0.25 in. diameter holes about 0.5 in. from each end on the same side and fit 2 pieces of 0.25 in. od × 2 in. copper pipe (part C) just into the holes and solder in place. Cut pipe with end caps lengthwise into 2 pieces as shown in Figure 1. Hold the 2 sections firmly together with two 2 or 1.5 in. hose clamps and drill holes, about 0.0625–0.125 in. greater than syringe barrel diameter, in center of each end. Dress all rough edges with suitable file.

To assemble mantle on syringe, slide 2 pieces of tygon or rubber tubing (part D) over syringe barrel and position them adjacent to end caps of mantle. Run bead of silicone sealant (e.g., Silicone seal, Canadian General Electric, Toronto, Ontario) along contacting edges of both sides of

Received December 2, 1981. Accepted February 22, 1982.

0004-5756/82/6505-1283-02$01.00

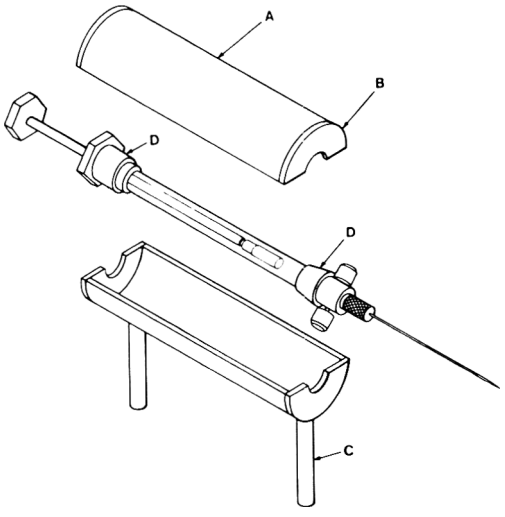


Figure 1. Metal syringe mantle, adapted for 1 mL Pressure-Lok® syringe: A, 1.5 in. id copper pipe cut lengthwise into 2 pieces; B, 0.0625 in. thick copper plate, one for each end; C, 0.25 od × 2 in. copper pipe, 2 pieces; D, rubber tubing gasket over metal end fittings of syringe.

mantle. Insert syringe and place the two 2 or 1.5 in. hose clamps around mantle just inside 0.25 in. tubes. Tighten hose clamps and smooth extruded silicone sealant.

Use

To heat syringe, thermostatically-controlled water can be recycled through mantle, or steam from boiling water bath can be drawn through mantle by using water aspirator. Alternatively, mantle may be connected to steam generator. Aspiration is preferred because steam or heating supply does not have to be turned off each time syringe is used. The hot mantle must be insulated, or the analyst must wear gloves for protection.

To connect mantle to steam or water supply, we use tygon tubing with ball joints or "quick disconnects" clamped in a stand. Mantle and syringe, with matching joint component, can then be readily connected or disconnected.

The use of steam or water as a heating source restricts mantle to a maximum of 100°C. Syringe can be removed from mantle after hose clamps are removed and silicone seal is slit with a razor blade. Because this mantle is easily made, in our laboratories we use a separate mantle for each size or brand of syringe. Using larger diameter rubber tubing (part D), a smaller diameter syringe, if long enough, may be reversibly fitted into a mantle designed for a larger syringe. To fit a larger syringe into the same mantle, the end holes would have to be irreversibly enlarged by drilling or filing.

The adaptation of this design of mantle to any syringe is limited by the diameter of the pipe (part A) and by the ease of sliding the rubber tubing (part D), which acts as a seal, onto the syringe barrel. Large protuberances at both ends of the syringe barrel would prevent the use of the rubber tubing seal. In the instance, split tubing, rubberized tape, or other compressible seals could be improvised.

The heating mantle described here is robust, easily fabricated from readily available parts, and, most important, can be made to fit syringes for which commercial mantles are unavailable.

Acknowledgments

The author thanks A. Trottier for the illustration and J. Perusse and staff for fabrication.

References

(1) Hachenberg, H., & Schmidt, A. P. (1977) *Gas Chromatographic Headspace Analysis*, Heyden and Son Ltd, London, UK
(2) Kolb, B. (Ed.) (1980) *Applied Headspace Gas Chromatography*, Heyden and Son Ltd, London, UK
(3) Drozd, J., & Novak, J. (1979) *J. Chromatogr.* **165**, 141-165

Investigations on Nonsulfonamide Bratton-Marshall-Positive Compounds in Porcine Liver

OWEN W. PARKS and WALTER I. KIMOTO
U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118

An unknown Bratton-Marshall-positive compound was observed when liver extracts were screened by a procedure for the simultaneous determination of sulfamethazine and sulfathiazole in swine livers. The unknown has been conclusively identified as *o*-aminobenzoic acid (anthranilic acid), a tryptophan metabolite, by gas-liquid chromatography-mass spectrometry of swine liver extracts obtained by a modified Tishler procedure for sulfonamide residues in animal tissues.

In a previous publication (1), we described a rapid thin layer chromatographic screening procedure for the simultaneous determination of sulfamethazine and sulfathiazole in swine livers. Application of this procedure to a number of liver samples resulted in the consistent observation of an unknown Bratton-Marshall-positive (2) compound. Although the unknown did not interfere with determining the presence or confirming the absence of the drugs, its potential was recognized for contributing to false positive results in the modified Tishler method (3) which is used by regulatory agencies for monitoring sulfonamide residues in swine livers. This communication presents evidence which identifies the unknown and establishes its presence in liver extracts obtained by a modified Tishler method.

METHOD

Reagents and Materials

(a) *Solvents.*—Ethyl acetate, dichloromethane (DCM), acetone, and methanol (Distilled-in-Glass®, Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442). Chloroform (Baker Analyzed reagent, J. T. Baker Chemical Co., Phillipsburg, NJ 08665).

(b) *Tetrabutylammonium hydroxide (TBAH).*—40% aqueous solution (Aldrich Chemical Co., Inc., Milwaukee, WI 53233).

(c) *Methylating reagent.*—0.2M iodomethane (Aldrich Chemical Co., Inc.) in DCM.

(d) *0.1M Carbonate buffer, pH 10.*—Prepared from 0.1M solutions of sodium carbonate and sodium bicarbonate.

(e) *N-(1-Naphthyl)ethylenediamine dihydrochloride (NEDA).*—Sigma Chemical Co., St. Louis, MO 63178. 0.4% methanol solution.

(f) *Benzoic acids and benzoates.*—*o*-Aminobenzoic acid, methyl *p*-aminobenzoate (Eastman Kodak, Rochester, NY 14650); *p*-aminobenzoic acid (Chem Service, Media, PA 19063); methyl *m*-aminobenzoate, methyl *o*-aminobenzoate (Chemalog, South Plainfield, NJ 07080).

(g) *Chromatographic support.*—100-120 mesh Chromosorb 102 (Sigma Chemical Co.).

Slurry 0.6 g in 10 mL methanol. Add slurry to super Pasteur disposable pipet (Curtin Matheson Scientific, Inc., Houston, TX 77001) plugged with glass wool. Add small wad of glass wool to top of column. Wash resin consecutively with 15 mL portions of methanol, acetone, and water before use. Note: Do not let column run dry.

(h) *Resin.*—Dowex-1, chloride form, 2% cross-linked, 200-400 mesh (Sigma Chemical Co.). Slurry sufficient resin in water to prepare 2 cm column in disposable Pasteur pipet plugged with glass wool. Add plug of glass wool to top of column and wash resin with 3 mL 1N NaOH. Rinse column with water until effluent is neutral, then wash column with 5 mL 1N HCl. Rinse column with water until effluent is neutral. Rinse column with 5 mL methanol before use.

Apparatus

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

(b) *Centrifuges.*—International clinical centrifuge, Rotor 213 (International Equipment Co., Needham Heights, MA 02194); Servall Superspeed centrifuge, type SS-1 rotor. (Ivan Sorvall, Inc., Norwalk, CT).

(c) *UV source.*—Chromato-Vue (Ultra-Violet Products, Inc., San Gabriel, CA 91778).

(d) *TLC plates.*—2.5 × 10 cm precoated (250 μm layer) silica gel G glass plates (Analtech, Inc., Newark, DE 19711). Free acid solvent system:

Received December 8, 1981. Accepted February 22, 1982.
Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

0004-5756/82/6505-1285-03$01.00

ethyl acetate–methanol (4 + 1); methyl ester system: hexane–ethyl acetate (3 + 1). Develop plates 0.5 cm from origin, dry in forced air oven at room temperature, and redevelop to height of 6 cm from origin. Partially air-dry developed plates and view under longwave (365 nm) UV light to locate blue-white fluorescent spot. Completely dry plates in forced air oven at room temperature and visualize aromatic primary amines with NEDA following diazotization (1).

(e) *GLC–MS system.*—70 eV electron impact mass spectra were obtained on Hewlett-Packard Model 5992-B combination GLC–low resolution quadrupole MS system interfaced to Hewlett-Packard Model 9825-A data system. GLC conditions: 1.83 m × 0.64 cm od glass column packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q; injection port 150°C; column programmed from 70 to 280°C at 6°/min; helium carrier gas flow rate 20 mL/min.

Liver Samples

Swine livers were obtained from a local slaughter house and portions were extracted within 3 h of being excised from the animals.

Procedures

(a) *Methylation of isolated unknown–TBAH ion pair.*—Combine DCM extracts from two 2.5 g liver samples obtained by screening procedure previously described (1). Evaporate combined extracts to dryness in 9 mL screw-cap specimen vial. Add 2 mL 0.2M iodomethane in DCM and stir mixture magnetically 30 min. Remove iodomethane-DCM under stream of nitrogen at 40°C. Dissolve residue in 0.4 mL DCM and spot 10 μL on thin layer chromatographic (TLC) plate. Develop plates in hexane–ethyl acetate (3 + 1).

(b) *Isolation of unknown by modified Tishler procedure* (3).—Add 20 mL chloroform–acetone (1 + 1) to 5 g swine liver in 50 mL polypropylene centrifuge tube and homogenize mixture 1 min at low speed. Centrifuge homogenate 2 min at 3500 rpm. Use Pasteur pipet to remove solvent, and filter solvent through plug of glass wool packed in super Pasteur pipet into 50 mL polypropylene screw-cap centrifuge tube. Just remove solvent under stream of nitrogen at 60°C and dissolve residue in 10 mL hexane. Add 2 mL 1N HCl and shake mixture 3 min in rocking motion and then centrifuge 2 min at 3500 rpm. Pour contents of centrifuge tube into 60 mL separatory funnel containing 8 mL water in manner that prevents mixing aqueous and organic phases. Recover acid phase (0.2N) and pass it through column of Chromosorb 102. Wash resin with 15 mL water and elute column with 5 mL methanol. Combine methanol effluents from 4 isolations (20 g liver) and evaporate to dryness in 9 mL screw-cap specimen vial under stream of nitrogen at 60°C. Dissolve residue in 0.5 mL methanol. Spot 10 μL aliquot on TLC plate and develop in ethyl acetate–methanol (4 + 1).

Carry out methylation by exhaustive alkylation procedure of Gyllenhaal et al. (4). Remove methanol under stream of nitrogen and add 2 mL pH 10 buffer and 0.05 mL TBAH solution to residue. Vortex-stir solution 30 s. Add 2 mL 0.2M iodomethane in DCM and magnetically stir mixture 4 h to complete methylation. Centrifuge reaction vial 2 min at 2500 rpm and slowly pour contents (less magnetic stirrer) into 60 mL separatory funnel containing 5 mL DCM. Rinse reaction vial with 6 mL DCM, centrifuge, and add solvent to separatory funnel. Recover DCM and evaporate it to ca 0.5 mL in 9 mL screw-cap specimen vial under stream of nitrogen. Spot 20 μL aliquot on TLC plate and develop plate in hexane–ethyl acetate (3 + 1). Evaporate sample to dryness.

Dissolve residue in 0.5 mL methanol and pass solution through Dowex 1 column, collecting effluent in 15 mL screw-cap centrifuge tube. Rinse vial with three 0.5 mL portions of methanol. Add rinses to ion exchange resin individually and collect total effluent. Remove methanol under stream of nitrogen at 50°C and dissolve residue in 10 mL DCM. Add 5 mL water and shake mixture vigorously 2 min. Centrifuge mixture 2 min at 2500 rpm, and then slowly add separated mixture to 60 mL separatory funnel containing 5 mL DCM. Recover DCM in 23 mL screw-cap specimen vial and evaporate to ca 3 mL at 40°C under stream of nitrogen. Transfer DCM by using disposable Pasteur pipet, in ca 0.5 mL increments, to 2 mL screw-cap specimen vial and evaporate to ca 75 μL at room temperature. Inject 15 μL into gas-liquid chromatographic-mass spectrometric (GLC-MS) system.

Results and Discussion

The unknown compound was tentatively identified in liver extracts obtained by the screening procedure which limits the extraction of Bratton–Marshall-positive compounds to amphoteric compounds (1). Hence, a variety of agricultural chemicals and drugs, and/or their metabolites (5), were eliminated as the possible source of the Bratton–Marshall-positive compound in the liver extracts. In addition, the

Table 1. Comparison of relative intensities (%) of the 10 most abundant mass fragment ions of methylated unknown Bratton–Marshall-positive compound with authentic methyl aminobenzoate isomers [a]

m/z	Unknown (10.6)[b]	*ortho-* (10.5)	*meta-* (13.6)	*para-* (15.2)
119	100.0	100.0	<1.0	<1.0
92	73.4	68.3	99.1	34.9
65	53.3	50.1	75.3	42.6
151	41.1	44.9	85.7	38.6
120	31.0	31.3	100.0	100.0
39	29.6	30.2	38.5	21.2
64	19.6	16.8	14.8	8.1
52	17.3	13.4	13.1	6.9
63	15.7	16.0	18.8	10.1
91	12.8	12.5	9.9	4.4

[a] Data corrected for background by data system.
[b] Values in parentheses are GLC retention times (min).

unknown was present to varying degrees in nearly all livers subjected to the screening procedure, thus suggesting to us that the unknown was a naturally occurring compound. Among such compounds, *p*-aminobenzoic acid (PABA) has been considered a potential source of false positive results in the Tishler procedure for sulfonamides (6). However, our comparative TLC studies of the unknown with PABA revealed 3 differences which eliminated this compound as the unknown: (a) R_f values were slightly different (PABA, 0.7; unknown, 0.6); (b) the unknown exhibited a blue-white fluorescence when viewed under longwave UV light whereas PABA did not fluoresce; and (c) the diazotized PABA reacted almost immediately with NEDA whereas the unknown developed color slowly.

Methylation of the isolated unknown-TBAH ion pair with iodomethane presented the first evidence as to the identity of the unknown. The methylated compound had a grape-like odor which we recognized as that of the *ortho*-isomer of aminobenzoic acid (anthranilic acid). TLC studies on authentic *o*-aminobenzoic acid and methyl *o*-aminobenzoate revealed the same R_f values, and the same fluorescence and color development characteristics with NEDA, as those for the unknown and its methyl ester. Based on this information, the unknown was tentatively identified as *o*-aminobenzoic acid.

This compound was also present in extracts obtained by the Tishler procedure. TLC results were positive on these extracts, and the identity of the unknown, as its methyl ester, was unequivocally established by GLC-MS. Table 1 presents GLC retention times and the relative intensities of the 10 most abundant MS ions of the methylated unknown and methyl *o*-aminobenzoate. The retention times and ion intensities of methyl *p*-aminobenzoate and methyl *m*-aminobenzoate are presented for comparison. Based on TLC and GLC-MS studies, we conclude that the unknown is *o*-aminobenzoic acid, a naturally occurring metabolite of tryptophan in animal tissues.

The specific contribution of *o*-aminobenzoic acid to the results obtained by the Tishler method for sulfonamide residues in swine liver is not precisely known. Our knowledge of the actual sampling, storage, and other conditions that occur before analysis in the regulatory monitoring program suggest that *o*-aminobenzoic acid contributes to the sulfa drug "background" level frequently encountered. Further investigations on this effect will be carried out.

References

(1) Parks, O. W. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 632–634.
(2) Bratton, A. C., & Marshall, E. K. (1939) *J. Biol. Chem.* **128,** 537
(3) Tishler, F., Sutter, J. L., Bathish, J. N., & Hagman, H. E. (1968) *J. Agric. Food Chem.* **16,** 50–53
(4) Gyllenhaal, O., Tjarnlund, U., Ehrsson, H., & Hartvig, P. (1978) *J. Chromatogr.* **156,** 275–283
(5) Phillips, W. F., & Traffon, J. E. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 44–47
(6) Lloyd, W. E., Jenny, A. L., Cox, D. F., & Rottinghaus, G. E. (1981) *Am. J. Vet. Res.* **42,** 339–343

FOR YOUR INFORMATION

1982 AOAC Wiley Award Winner is Odette Shotwell, U.S. Department of Agriculture

Odette L. Shotwell, Research Leader, Mycotoxin Analytical and Chemical Research Unit, Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois, is the winner of the 1982 Harvey W. Wiley Award. James P. Minyard, 1982 AOAC President, will present the award to Dr. Shotwell on Monday evening, October 25, at the AOAC 96th Annual International Meeting.

The $2500 award is given annually to a scientist who has made outstanding contributions to the development and validation of methods of analysis for foods, drugs, cosmetics, pesticides, feeds, fertilizers, environmental contaminants, or other related areas. The award was established in 1956 in honor of Harvey W. Wiley, "Father" of the 1906 Pure Food and Drug Act and a founder of AOAC. Its primary purpose is to emphasize the role of the scientist in protecting the consumer and the quality of the environment.

Dr. Shotwell has conducted and led research at the National Regional Research Center for 34 years. Since 1965, the focus of her work has been on mycotoxins, particularly aflatoxins and Fusaria toxins. Through this continuous research, she and her group have played a key role in providing, improving, and validating analytical methodology to protect workers and consumers from mycotoxins and have produced 11 distinct discoveries and developments used in agriculture, industry, government, and universities.

Mycotoxins are not Dr. Shotwell's only area of expertise. For 12 years, she investigated the chemistry of antibiotics and was involved in the discovery and characterization of 4 new antibiotics—hydroxystreptomycin, cinnamycin, curamycin, and azacolutin. In addition, as senior chemist, over a 10-year period, she supervised studies of Japanese beetle hemolymph in a program on microbiological control of the beetles by milky spore disease organisms. These years of research are documented by 138 publications and 87 presentations including 40 invited papers.

Odette Shotwell's achievements and expertise have not gone unrecognized. She received the U.S. Department of Agriculture's Distinguished Service Award and its Certificate of Merit, and has been a member or chairman of various regional, national, and international mycotoxin committees. She is a consultant to the Food and Drug Administration's Bureau of Veterinary Medicine, and a member of the Editorial Board of the *Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes.*

Active in various professional organizations, Dr. Shotwell has been an AOAC Associate Referee for Mycotoxins in Grains since 1968 and was elected a Fellow of the AOAC in 1978. She is a member of the American Oil Chemists' Society Mycotoxin Committee, chairman of the American Association of Cereal Chemists Mycotoxin Committee, a member of the Joint Association of Official Analytical Chemists/American Oil Chemists' Society/American Association of Cereal Chemists/International Union of Pure and Applied Chemistry Mycotoxin Committee, a member of the American Oil Chemists' Society Smalley Mycotoxin Sample Committee, secretary of the Regional NC-151 Committee "Marketing and Delivery of Quality Cereals and Oilseeds in Domestic and Foreign Markets," and chairman of an NC-151 subcommittee.

0004-5756/82/6505-1288-05$01.00

Dr. Shotwell holds a B.S. degree from Montana State University which awarded her their Distinguished Alumni Award in 1961. Her M.S. and Ph.D. degrees are from the University of Illinois.

Dr. Shotwell is a resident of Peoria, Illinois, where she is deeply involved in community affairs. She is active as Chairman of the NAACP Education Committee on the improvement of educational opportunities for minorities and underprivileged, is a commissioner of the Mayor's Advisory Board for the Handicapped, chaired the Informational Branch of the Peoria Human Relations Committee, was President of the Peoria Chapter of the League of Women Voters, and was instrumental in planning and fund raising for the Lakeview Center for the Arts and Sciences.

AOAC to Hold Its 96th Annual International Meeting October 25-28, 1982

The Association of Official Analytical Chemists (AOAC) will hold its 96th Annual International Meeting October 25-28, at the Shoreham Hotel, Washington, DC.

The latest in analytical methodology in agricultural, environmental, and public health areas will be presented and discussed. The meeting will include the following 5 symposia: Measurement of Water Activity in Foods, Tuesday, October 26; Advances in Nitrosamine Analysis, Tuesday, October 26; Chemical Analysis of Drinking Water—Trace Contaminants, Wednesday, October 27; Good Laboratory Practices, Wednesday, October 27; and Detection of Deliberate Adulteration of Foods, Thursday, October 28.

About 200 papers will be given on new techniques, methods and instrumentation, analysis of foods, drugs, pesticides, cosmetics, feeds, fertilizers, mycotoxins, beverages, colors, forensic science materials, hazardous substances, vitamins, water and air pollutants, microbiological and extraneous materials, contamination of foods, and related material.

October 22-24, immediately preceding the AOAC Meeting, the American Chemical Society will sponsor a short course, "Practice of Modern Liquid Chromatography." Instructors will be Dr. J. J. Kirkland of E. I. duPont de Nemours and Co., and Dr. Lloyd R. Snyder of Technicon Instrument Corp.

Evening seminar workshops will be held October 26th by LaChat Instruments on automated analysis, October 26th by Tecator, Inc. on a topic to be decided, and October 27th by Technicon Industrial Systems on automated analysis.

For further details contact Kathleen M. Fominaya, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209; telephone 703/522-3032.

1982 Fellows of the AOAC

Eight scientists have been chosen to receive the 1982 Fellow of the AOAC awards. James P. Minyard, AOAC President, will make the presentations at the opening session of the 95th Annual International Meeting of the AOAC, on Monday, October 25, 1982.

The award recipients are Wallace S. Brammell, Food and Drug Administration, Washington, DC; D. Earle Coffin, Health and Welfare Canada, Ottawa, Ontario, Canada; Thomas Fazio, Food and Drug Administration, Washington, DC; Elmer George, Jr, New York Department of Agriculture and Markets, Albany, NY; Arthur H. Hofberg, Ciba-Geigy Corp., Greensboro, NC; Stanley E. Katz, Rutgers University-Cook College, New Brunswick, NJ; John W. Sherbon, Cornell University, Ithaca, NY; and Edward Smith, Food and Drug Administration, Washington, DC.

The Fellow of the AOAC award was established in 1961 to recognize those persons giving meritorious service to the Association. Winners of the Award have performed notably for 10 years or more, usually as officers, referees, or committee members. Nominations are made by AOAC members, reviewed by the Committee on the Fellows, and finally approved by the Board of Directors.

Wallace S. Brammell has a total of 4 Associate Refereeships and 1 General Refereeship. At present, he is Associate Referee on Phosphorus in Eggs and Egg Products, Sodium Chloride in Vegetable Products, Phosphorus in Dairy Products, and Inorganic Salts in Colors, and is General Referee on Eggs and Egg Products.

D. Earle Coffin has served on 4 Committees and the Board of Directors. Of these, he is presently a member of the Board and of Committee C, and is Chairman of the Committee on the Constitution.

Thomas Fazio has served as Associate Referee on 2 topics in the past and is, at present, General Referee on Food Additives, Chairman of the Committee on Nitrosamines,

and AOAC Representative to ICC Committee on Additives and Residues.

Elmer George, Jr, has served on a total of 3 Committees and on the Official Methods Board. He is presently Chairman of the latter and a member of Committee D.

Arthur H. Hofberg has served as a member of a Committee and as Associate Referee on a total of 9 topics. Of the 9, he is, at present, Associate Referee on 7: s-Triazine Herbicides, Diazinon, Fluometuron, Chloroxuron, Chlorodimeform, Metolachlor, and Larvadex.

Stanley E. Katz has served and is presently serving as Associate Referee on Slow Release Mixed Fertilizers and on Tetracyclines in Tissues, as General Referee on Antibiotics, as a member of the Committee on State Participation and the Committee on Collaborative Studies, and as Chairman of the Ways and Means Committee.

John W. Sherbon has served and is, at present, serving as Associate Referee on the following 3 topics: Rapid Tests for Solids-Not-Fat in Dairy Products, Rapid Tests for Protein in Milk, and Enzymatic Determination of Lactose in Dairy Products.

Edward Smith has served as Associate Referee on 2 topics, Glutethimide, and Opium and Paregoric and is serving as General Referee on Alkaloid Drugs and as a member of the Committee on Collaborative Studies.

AOAC's Private Sustaining Membership Grows

AOAC welcomes 2 new additions to the growing list of companies aware of the need to support an independent methods validation association. These new members are Gerber Products Co., Fremont, MI, and Nabisco Brands, Inc., Fair Lawn, NJ.

In the Author's Best Interest – An Editor's Advice

From time to time editorials are written on the subject of preparation of manuscripts submitted for publication, and on the time that elapses between receipt of a paper and its eventual publication. Very often a great deal of delay can be caused by poor preparation of the original manuscript, especially with regard to the drawing of figures and the typing of references. If figures are badly drawn or the lettering on them is untidy, it is necessary to have them redrawn and relettered, and this not only takes time in the drawing office, but may also lead to error if the draughtsman concerned is unable to decipher or interpret the original correctly. With a set of good quality stencils, a drawing board and a T-square, or alternatively a good typewriter with a carbon film ribbon, lettering should be no problem. Likewise a set of French curves or a flexible ruler should enable the author to produce acceptably smooth curved lines on graphs, etc. The size of letters and thickness of line to use will depend on the size of the drawing and the degree of reduction to be used. Measurement of the lettering on a published figure of good quality, in relation to the scale of the figure, should give a good idea of the letter size to use, and the thickness of the stencilled letter will indicate the line thickness needed for the drawing itself.

There is no excuse whatever for carelessness in listing references, since no skill is needed to copy character by character from the original. A conscientious editor may check not only the "suspicious" references, but the whole lot, and this can take a great deal of time, especially in the case of a review paper, and if some of the references are found to be non-existent or to have nothing whatever to do with the paper, the editor will either try to find the correct references to save delay in producing the paper, or be obliged to write to ask the author to do the job. In either case his faith in the scientific work in the paper is bound to be somewhat reduced, and he can scarcely be blamed if he feels annoyed at having to waste time that could be more rewardingly employed. The editor's duty is to protect the reader from false information, and at the same time to protect the author from having his carelessness made public for all to see, but he is entitled to expect authors not to give him occasion to perform this duty. He is also entitled to expect authors to prepare their references in the style used by *his* journal and not that of another journal. It is in the author's own interests to do this anyway, since a referee may well suspect that a paper has been already rejected by the journal that uses the reference style employed by the author! It is also important that references should be checked against the original journal whenever possible—the abstracting journals are not always correct in the details given. Furthermore, the abstracting journals (with the honourable exception of *Zeitschrift für Analytische Chemie*) omit any accents on authors' names, which is discourteous as well as inaccurate.

We therefore urge all authors, whether contributing to this or another journal, to act in their own best interests by taking proper care in preparing their manuscripts. The editor will deal with any linguistic problems, and some editors will do their best to make sure the chemistry is correct as well, but responsibility for the figures and references lies primarily with the author(s).

—Reprinted from *Talanta*, Vol. 28, p. 1, 1981 with permission of Pergamon Press Ltd.

AOCS Appoints Its First Director of Methods Development

James "Scotty" Miller has been named the first Director of Methods Development for the American Oil Chemists' Society. AOCS is a professional society whose members work in the chemistry, biochemistry, nutrition, or processing of fats, oils, lipids, and protein. Miller will coordinate the modernization and expansion of the AOCS Book of Official and Tentative Methods.

Most recently director of laboratories of the Research Institute of the University of Petroleum and Minerals at Dhahran in Saudi Arabia, Miller has also worked as division manager of laboratory services with the Illinois Environmental Protection Agency, as vice president of Trace Elements, Inc., as an atomic absorption specialist with Perkin-Elmer, as chief chemist for the state of Alaska, and as a chemist with Boeing Co. and E. I. DuPont Co.

SRMs for Citrus Leaves and Bovine Liver Will be Available This Winter

SRM 1572 Citrus Leaves—A new botanical Standard Reference Material, SRM 1572 certified for chemical composition and intended as a replacement for SRM 1571, Orchard Leaves, will soon be available. The botanical SRM series is useful for assessing the accuracy of elemental determinations in plant tissues, agricultural food products, and similar natural matrices of environmental and nutritional interest, and for calibrating the instruments used in these analyses.

The Certificate of Analysis for SRM 1572 lists certified concentrations and their uncertainties for the elements calcium, magnesium, phosphorus, and potassium, and for trace elements aluminum, arsenic, barium, cadmium, chromium, copper, iron, lead, manganese, mercury, molybdenum, nickel, rubidium, sodium, strontium, and zinc.

Two other plant tissue SRMs are available in this series—SRM 1573, Tomato Leaves, and SRM 1575, Pine Needles. These Standard Reference Materials may be purchased at the following prices: 1571 at $49 per 10-g unit and 1572, 1573, or 1575 at $92 per 70-g unit, from the Office of Standard Reference Materials, Room B311, Chemistry Bldg, National Bureau of Standards, Washington, DC 20234; telephone 301/921-2045.

SRM 1577a Bovine Liver—Standard Reference Material 1577a, also available this winter, is a renewal of the previously issued Bovine Liver (SRM 1577). This animal tissue certified reference material was developed for use in validating methods for the analysis of animal tissues and other biological matrices, and for calibrating instruments used in these analyses. These methods are of importance in the food industry and in medical studies.

The Certificate of Analysis for SRM 1577a provides certified concentrations and their uncertainties for the following elements: arsenic, calcium, chlorine, cobalt, iron, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium, zinc, cadmium, lead, mercury, rubidium, silver, strontium, and uranium. The following elements are not certified, but are given for information only: aluminum, antimony, bromine, nitrogen, sulfur, and thallium.

SRM 1577a is a freeze-dried material prepared from fresh bovine liver. It may be purchased for $107 per unit of 50 g (3 bottles, each containing approx. 17 g) from the Office of Standard Reference Materials, Room B311, Chemistry Bldg, National Bureau of Standards, Washington, DC 20234; telephone 301/921-2045.

Cross Reference Service for Gas Analysis

Scott Specialty Gases has released a new bulletin describing their expanded Cross Reference Services, a program now in its 18th year. Samples of unknown concentrations are provided to participants for analysis. Scott statistically evaluates the results so participating laboratories can measure the analytical accuracy they have achieved. Components in the gas mixtures provided include oxygen, sulfur dioxide, carbon monoxide, oxides of nitrogen, hexane, benzene, and other hydrocarbons. The price of the quarterly services ranges from $325 to $430 per service per year. For free bulletin,

contact Scott Specialty Gases, Plumsteadville, PA 18949; 215/766-8861.

Meetings

September 23-24, 1982: 5th European Seminar on Quality Control in the Pharmaceutical and Cosmetic Industries—Administrative and Economic Problems, University of Geneva, Switzerland. Contact: Economics of Quality Control Pharma Cosmetic Section, c/o SAQ, PO Box 2613, CH-3001 Berne, Switzerland; Telex 33528 atag ch; telephone 031 22 03 82.

October 12-14, 1982: Dioxin 82—3rd International Symposium/Workshop on Chlorinated Dioxins and Related Compounds, International Conference Centre, Salzburg, Austria. Contact: E. Merian, Im Kirsgarten 22, CH—4106 Therwil, Switzerland.

October 18-21, 1982: ISA/82 International Conference and Exhibit, Philadelphia Civic Center, Philadelphia, PA. Contact: Steve Settle, Instrument Society of America, 67 Alexander Dr, Research Triangle Park, NC 27709; 919/549-8411.

October 22-23, 1982: 8th Annual Meeting of the Northeastern Association of Forensic Scientists, Sheraton Airport Inn, Albany, NY. Contact R. C. Herrmann, State Police Headquarters Crime Laboratory, State Campus, Bldg. 22, Albany, NY 12226; 518/457-1208.

October 24-28, 1982: American Association of Cereal Chemists 67th Annual Meeting, San Antonio, TX. Contact: AACC, 3340 Pilot Knob Rd, St. Paul, MN 55121; 612/454-7250.

October 25-28, 1982: 96th AOAC Annual International Meeting, Shoreham Hotel, Washington, DC. For information, see article above.

December 6-8, 1982: Third Bi-annual International Thin Layer Chromatography Symposium, Hilton Hotel, Parsippany, NJ. Contact: J. C. Touchstone, Hospital University of Pennsylvania, Philadelphia, PA 19104; 215/662-2081; or H. M. Stahr, 1636 College of Veterinary Medicine, Iowa State Univ., Ames, IA 50011; 515/294-1950.

February 13-17, 1983: International Conference on Oils, Fats & Waxes, University of Auckland, Auckland, New Zealand. Contact: S. G. Brooker, International Conference on Oils & Fats, Chemistry Dept, University of Auckland, Private Bag, Auckland, NZ.

May 24-26, 1983: 37th Annual Quality Congress and Exposition, Boston, MA. Contact: Holly A. Kuusinen, American Society for Quality Control, 230 West Wells St, Milwaukee, WI 53203; 414/272-8575.

June 7-10, 1983: 1st International Symposium on Drug Analysis, Free University of Brussels, Brussels, Belgium. Contact: C. Van Kerchove, Société Belge des Sciences Pharmaceutiques—Belgisch Genootschap voor Pharmaceutische Wetenschappen, rue Archimedesstraat 11, B-1040 Brussels, Belgium; telephone (02) 733 98 20 ext. 33.

July 17-23, 1983: SAC 83—International Conference and Exhibition on Analytical Chemistry, the University of Edinburgh, Edinburgh, Scotland. Contact: P. E. Hutchinson, Secretary, Analytical Div., Royal Society of Chemistry, Burlington House, London, W1V OBN, UK.

July 27-30, 1983: 3rd International Conference on Instrumental Analysis of Foods and Beverages—Recent Developments in Chemistry and Technology, Corfu Hilton Hotel, Corfu, Greece. Contact: D. J. Mussinan, IFF R & D, 1515 Highway 36, Union Beach, NJ 07735; 201/264-4500.

Course Offered

The Rocky Mountain Center for Occupational and Environmental Health at the University of Utah, Salt Lake City, Utah, is offering a course entitled "Industrial Hygiene Chemistry (NIOSH 590)," November 15-19, 1982. Tuition is $500.00. for further information contact: RMCOEH, Ms K. Blosch, Univ. of Utah, Building 512, Salt Lake City, UT 84112; telephone 801/581-5710.

BOOK REVIEW

Modern Fluorescence Spectroscopy. Volumes 3 and 4. Edited by E. L. Wehry. Published by Plenum Press, Division of Plenum Publishing Corp., 233 Spring St, New York, NY 10013, 1981. Vol. 3: approx. 350 pp., price $39.50, hardcover, ISBN 0-306-40690-X; Vol. 4: approx. 275 pp., price $35, hardcover, ISBN 0-306-40691-8.

These volumes are a continuation of a series which covers advances in fluorescence techniques. Each chapter is written by one or more authors and covers a different aspect of luminescence technology which is now commercially available as well as speculations on future technology. There is something of interest for a wide range of readers. Some topics will be of interest to biologists and others to bench analysts and those working in method development in industry or regulatory agencies. There are also theoretical chapters for the academician. The contributors are from the United States and Europe. The content of Volume 3 tends toward practical application while that of Volume 4 is more theoretical.

Volume 3 covers such diverse topics as quantitation of DNA and RNA in a single cell and identification of mixtures from their fluorescence spectrum through a computerized search routine. There are practical tips on the use of fluorescence detectors for both liquid and gas chromatographic analyses and a discussion of pre-column and post-column reactions to enhance analyte fluorescence and thereby sensitivity. The chapter on fluoroimmunoassay provides an overview of immunoassay techniques as well as a comparison to radioimmunoassay. Analysis of complex samples through kinetic measurement is also covered. Where appropriate, each chapter includes a discussion of the instrumentation used by that specific technique.

Volume 4 discusses the newest technological advancements such as, the use of lasers as excitation sources, the development of array detectors to produce excitation-emission matrices (EEM) for three-dimensional plots and the use of these EEM plots to analyze mixtures, synchronous excitation spectroscopy and its applications, and identification of oil spills by fluorescent spectral manipulation and by a low-temperature technique. The reader needs a good background in fluorescence theory to understand several of the chapters in this volume.

Both volumes contain interesting and pertinent topics. Improvement in instrumentation has enhanced the sensitivity and selectivity of fluorescence spectroscopy. Modern fluorescence techniques offer the chemist greater capability in the analysis of multicomponent mixtures at lower levels.

ELAINE A. BUNCH

Seattle Field Office
Food and Drug Administration
Seattle, WA 98174

NEW PUBLICATIONS

Dairy Microbiology. Volume 1 – The Microbiology of Milk & Volume 2 – The Microbiology of Milk Products. Edited by R. K. Robinson. Published by Applied Science Publishers Ltd, 22 Rippleside Commercial Estate, Ripple Rd, Barking, Essex 1G11 OSA, England, 1981. Volume 1: x + 255 pp., 37 illus., price US $38 plus $4 post/packing, ISBN 0-85334-948-7; Volume 2: ix + 330 pp., 31 illus., price US $46 plus $4 post/packing, ISBN 0-85334-961-4.

Volume 1 contains chapters on milk and milk processing, microorganisms associated with milk, control and destruction of microorganisms, the microbiology of raw milk, market milk, dried milk powders, and concentrated milks.

Volume 2 contains chapters on the microbiology of ice cream and related products, cream and dairy desserts, butter, starter cultures, cheese, and fermented milks, and on quality control in the dairy industry.

Reversed-Phase High-Performance Liquid Chromatography – Theory, Practice, and Biomedical Applications. By A. M. Krstulovic and P. R. Brown. Published by Wiley Professional Books-By-Mail, Dept 0001, Somerset, NJ 08873, 1982. 312 pp. Price $35.00. ISBN 1-05369-4.

Reversed-phase liquid chromatography (RPLC) has increased tremendously in popularity over the past 10 years. This book covers both theoretical aspects of RPLC and practical information needed in diverse areas of research. It also contains a review of the RPLC applications in the biomedical/biochemical fields, with references and collateral readings.

Recent Developments in Food Analysis – Proceedings of the First European Conference on Food Chemistry. Edited by W. Baltes, P. B. Czedik-Eysenberg, and W. Pfannhauser. Published by Verlag Chemie International Inc., Plaza Centre, Suite E, 1020 NW 6th St, Deerfield Beach, FL 33441, 1982. 550 pp., 192 figures, 94 tables. Price $61.30. Paperback. ISBN 3-527025942-2.

The lectures and posters that were presented at the First European Congress on Food Chemistry (EURO FOOD CHEM I) are presented in this book. It provides reports on 67 topics of current importance in food analysis. The methods include chromatography, mass spectrometry, electrophoresis, immunological and enzymatic assays, as well as procedures for the analysis of inorganic contaminants. Applications of these methods in the analysis of a variety of foodstuffs are described.

Analysis of Pesticides in Water. Volume 1: Significance, Principles, Techniques, and Chemistry of Pesticides. Edited by A. S. Y. Chau. Published by CRC Press, Inc., 2000 NW 24th St, Boca Raton, FL 33431, 1982. 224 pp. Price $69.50 in U.S., $80 outside U.S. ISBN 0-8493-5210-X.

Volume 1 is a survey of the analytical methodology used in the analysis of pesticides. Essential background information emphasizing practical aspects of various methods is given. Advantages and disadvantages of methods are examined, and the experiences of the author are discussed.

Laboratory Decontamination and Destruction of Aflatoxins B_1, B_2, G_1, G_2 in Laboratory Wastes. Edited by M. Castegnaro, D. C. Hunt, E. B. Sansone, P. L. Schuller, M. G. Siriwardana, G. M. Telling, H. P. Van Egmond, and E. A. Walker. Published by the International Agency for Research on Cancer, 1980. Available from WHO Publications Centre USA, 49 Sheridan Ave, Albany, NY 12210. 59 pp. Price: Sw.fr. 18.0, US $10. IARC Scientific Publications No. 37.

The first of a series, this volume deals with laboratory decontamination and destruction of aflatoxins in laboratory wastes. Of the seven methods investigated, 4 are recommended. These 4 methods are described in International Organization for Standardization (ISO) format and cover disposal of stock, aqueous, and nonaqueous solutions, content of Petri dishes, animal carcasses and bedding, and decontamination of work surfaces, clothing, equipment and accidental spills. An extensive bibliographical review of the chemical and

biological properties of these aflatoxins is also included.

McGraw-Hill Encyclopedia of Science and Technology – Fifth Edition. Editor-in-Chief Sybil P. Parker. Published by McGraw-Hill Book Co., 1221 Avenue of the Americas, New York, NY 10020, 1982. 15 volumes, 15 000 pp. + illus. Price: $850.00. ISBN: 0-070079280-1.

To reflect the scientific and technical achievements of the 5 years since the previous edition was published, this edition has been extensively revised and updated. Entries on 75 separate disciplines in science and technology examine the physical and natural sciences and engineering disciplines and discuss basic and recent theories, concepts, terminology, discoveries, materials, methods, and techniques. New material is included on genetic engineering, artificial intelligence, nuclear medicine, desertification, psycholinguistics, industrial robots, and immunoassay, as well as, video disk recording, metallic glasses, acoustic levitation, magnetic bubble memory, gluons, and computerized tomography.

Food Packaging Materials: Aspects of Analysis and Migration of Contaminants. By. N. T. Crosby. Published by Applied Science Publishers Ltd, 22 Rippleside Commercial Estate, Ripple Rd, Barking, Essex 1G11 OSA, England, 1981. 188 pp., 21 illus. Price: US $28 plus $4 post/packing. ISBN 0-85334-926-6.

Contents: 1. Food packaging requirements. 2. Plastic packaging materials. 3. Determination of monomers. 4. Toxicological aspects. 5. International legislation. 6. and 7. Migration—theoretical aspects and experimental determination. 8. Other food contact materials.

Reactive Polymers – Ion Exchangers Sorbents. Editor-in-Chief, F. G. Helfferich. Published by Elsevier Scientific Publishing Co., PO Box 330, 1000 AH, Amsterdam, The Netherlands. Also available from Elsevier North-Holland Inc., 52 Vanderbilt Ave, New York, NY 10017. Published quarterly beginning July 1982. Subscription price per volume (4 issues): US $76/Dutch Guilders 190.

Reactive Polymers is a new journal devoted to science and technology of polymers with functional groups providing chemical or physical reactivity. The scope extends from inorganic zeolites to organic resins, functioning as ion exchangers, selective sorbents, catalysts, biologically active reagents, etc. and their applications such as water or effluent treatment, separation, recovery, catalysis, or organic synthesis. It is for both the research worker and the practicing engineer, and its audience should include chemists, chemical engineers, pharmaceutical chemists, agricultural chemists, metallurgists, biochemists. biophysicists, and environmental engineers.

OBITUARY

Ernest A. Epps, Jr

Ernest Allen Epps, Jr, a former president of the AOAC (1976–1977), died July 28, 1982. Ernie, as he was known by all, was born March 18, 1917 in Corinth, Mississippi and received his BS in chemistry from Louisiana Polytechnic Institute in 1936 and an MS in soils and chemistry from Louisiana State University in 1938. After serving as an instructor in Agronomy at Louisiana State University, a soils technologist with the US Bureau of Reclamation, and as a chemist with Bay Chemical Company and Standard Oil of New Jersey, he assumed the Chief Chemist position of the Louisiana Department of Agriculture and the Louisiana Agricultural Experiment Station in 1946, positions which he occupied for almost 35 years, until his retirement.

Ernie served as the AOAC president in 1975–1976, after serving as President of the three related Control Official Societies—Association of American Pesticide Control Officials, Association of American Feed Control Officials, and Association of American Fertilizer Control Officials. During his tenure, the AOAC held its first annual Spring Workshop and Training Conference and distributed the FDA *Bacteriological Analytical Manual*, 4th edition, and the EPA *Manual of Chemical Methods for Pesticides and Devices*. He served the AOAC as General Referee on Feeds for over 15 years and published more than 25 papers on agricultural subjects, many in the AOAC *Journal*.

Although Ernie used automated instruments, in his Presidential address he pointed out that most automated methods are merely automated finishes. He stated that with all his instruments and computers, the laboratory was not doing more work or better work, and that the chemist was still the important feature of the analytical laboratory. Ernie, as the State Chemist of Louisiana, continued the important contributions made to the AOAC by the chemical laboratories and experiment stations of the states.

WILLIAM HORWITZ

INSTRUCTIONS TO AUTHORS

Scope of Articles

The Journal of the AOAC will publish articles that present, within the fields of interest of the Association (*a*) unpublished original research; (*b*) new methods; (*c*) further studies of previously published methods; (*d*) background work leading to development of methods; (*e*) compilations of authentic data; (*f*) technical communications, cautionary notes, and comments on techniques, apparatus, and reagents; (*g*) invited reviews of methodology in special fields. All articles are reviewed for scientific content and appropriateness to the journal.

Preparation of Manuscript

Authors are required to submit three copies of the complete manuscript, including all tables and all illustrations. The manuscript is to be typewritten on one side only of white bond paper, $8\frac{1}{2} \times 11$ inches, with minimum page margins of 1 inch, and must be **double-spaced** throughout (including title, authors' names and addresses, footnotes, tables, references, and captions for illustrations, as well as the text itself). Tables are to be typed on separate sheets, *not* interspersed through the manuscript. Drawings and photographs should be mounted apart from the text or submitted as separate items, *not* interspersed through the text.

Style and Format

The text should be written in clear, concise, grammatical English. Unusual abbreviations should be employed as little as possible and must always be defined the first time they appear. Titles of articles should be specific and descriptive. Full first names, middle initial (if any), and last names of authors should be given. The address of the institution (including zip code) from which the paper is submitted should be given and should be in a form to which inquiries, proofs, and requests for reprints can be sent. Information supplementing the title and authors' names and addresses should be given in footnote form.

Methods, Results and/or Discussion, Acknowledgments, and Recommendations (applicable to reports of General and Associate Referees) should be placed in separate sections under appropriate headings typed in capitals and lower case letters, centered on the page, *not* underscored.

Abstracts: Each manuscript should be accompanied by a concise abstract (not more than 200 words). The abstract should provide specific information rather than generalized statements.

Introduction: Each article should include a statement on why the work was done, the previous work done, and the use of the compound being studied.

Methods: Methods should be written in imperative style, i.e., "Add 10 mL . . . Heat to boiling . . . Read in spectrophotometer." Special reagents and apparatus should be separated from the details of the procedure and placed in sections with appropriate headings; however, common reagents and apparatus (e.g., concentrated HCl, chloroform, ordinary glassware, ovens, etc.), or those which require no special preparation or assembly, need not be listed separately. Hazardous and/or carcinogenic chemicals should be noted. The steps of the procedure should not be numbered, but should be grouped together to form a logical sequence of two, three, or four operations. Any very long, detailed operation can be given in a separate section with an appropriate heading (e.g., Preparation of Sample; Extraction and Cleanup; Preparation of Standard Curve). Any necessary calculations should be included. Care should be taken that the number of significant figures truly reflects the accuracy of the method. Equations should be typed in one-line form. Wherever possible, metric units should be used for measurements or quantities.

Tables: All tables must be cited in the text consecutively. Tables are numbered by arabic numbers, and every table must have a descriptive title, sufficient so that the table can stand by itself without reference to the text. This title should be typed in lower case letters, *not* capitals, with the exception of the word "Table" and the first word of the descriptive portion of the title, of which the first letter is capitalized. Every vertical column in the table should have a heading; abbreviations may be used freely in the headings to save space, but should be self-evident or must be explained in footnotes. Footnotes to both the headings and the body of the table are indicated by lower case letters in alphabetical order; these letters should be underscored and raised above the line of type. Horizontal rules should be used sparingly; however, they are used to bound the table at top and bottom and to divide the heads from the columns. Authors should refer to recent issues of the Journal for acceptable format of tables; tables should not exceed the normal page width of the Journal, and authors should attempt to revise or rearrange data to fit this pattern.

Illustrations: Illustrations, or figures, may be submitted as original drawings or photographs; photocopies are acceptable for the two review copies but not for the printer's copy. All figures must be cited in the text consecutively. Figures are numbered by arabic numbers, and all figures must be accompanied by descriptive captions, typed on one (or more) separate sheets, *not* on the figure itself. The figure should be identified by number on the back by a soft pencil or (preferably) a gummed label.

Drawings should be submitted either as the original drawing or a good glossy photograph; photocopies, multiliths, Verifax copies, Xerox copies, etc. are *not* acceptable. Drawings should be done in black India ink (ordinary blue or blue-black ink is not acceptable) or with drafting tape on white tracing paper or tracing cloth or on "fade-out" graph paper (ordinary graph paper ruled with green or dark blue ink is not acceptable). Lettering should be done with a Leroy lettering set, press-on lettering, or a similar device; freehand or

typewritten lettering is not acceptable. Values for ordinate and abscissa should be given, with proper identification conforming to journal style (example: wavelength, nm), at the sides and bottom of the figure. Lettering or numbering on the face of the figure itself should be kept at a minimum; supplementary information should be given in the caption. Several curves on the same figure should be identified by simple symbols, such as letters or numbers, and the proper identification or explanation given in the caption. Letters and numbers should be large enough to allow reduction to journal page or column size. *JAOAC* does not publish straight line calibration curves; this information can be stated in the text. The same data should not be presented in both tables and figures.

Footnotes: Footnotes are a distraction to the reader and should be kept to a minimum. Footnotes to the text are identified by arabic numbers set above the line of type (not asterisks or similar symbols). Each footnote must be indicated by its number within the text.

Acknowledgments: Essential credits may be included at the end of the text but should be kept to a minimum, omitting social and academic titles. Information on meeting presentation, financial assistance, and disclaimers should be unnumbered footnotes and appear after the *References* section.

References: References to previously published work should be collected at the end of the article under the heading "References." Each item in the list is preceded by an arabic number in parentheses. Every reference must be cited somewhere in the text in numerical order (rather than alphabetical or chronological). (*Note:* If an article contains only one reference, this reference may be inserted directly in the text, rather than placed at the end.) It is the author's responsibility to verify all information given in the references.

References to journal articles must include the following information: last names and at least one initial of *all* authors (*not* just the senior author); year of publication, enclosed in parentheses; title of journal, abbreviated according to accepted *Chemical Abstracts* style; volume number; numbers of first and last pages. References to books, bulletins, pamphlets, etc. must include the following information: last names and initials of authors or editors; year of publication, enclosed in parentheses; full title of book; volume number or edition (unless it is the first edition); publisher; city of publication; numbers of pertinent pages, chapter, or section. Citation to private communications or unpublished data should be included in the text, *not* in the list of references, in the following form: author's name and affiliation, and year.

The abbreviation for the journal title should be repeated for each reference; do not use *ibid.* *This Journal* will be referred to as *J. Assoc. Off. Anal. Chem.*

The compendium of methods of the Association shou.d be listed as follows: *Official Methods of Analysis* (198C) 13th Ed., AOAC, Arlington, VA, with appropriate section numbers; the edition and year are, of course, subject to change.

Symbols and Abbreviations

kg	kilogram(s)
g	gram(s)
mg	milligram(s)
μg	microgram(s)
ng	nanogram(s)
L	liter(s)
mL	milliliter(s)
μL	microliter(s)
m	meter(s)
cm	centimeter(s)
mm	millimeter(s)
μm	micrometer(s) (*not* micron)
nm	nanometer(s) (*not* millimicron)
A	ampere(s)
V	volt(s)
dc	direct current
ft	foot (feet)
in.	inch(es)
cu. in.	cubic inch(es)
gal.	gallon(s)
lb	pound(s)
oz	ounce(s)
ppm	parts per million
ppb	parts per billion
psi	pounds per square inch
sp gr	specific gravity
bp	boiling point
mp	melting point
id	inside diameter
od	outside diameter
h	hour(s)
min	minute(s)
s	second(s)
%	percent
⅌	standard taper
N	normal
M	molar
mM	millimolar

(Note: Spectrophotometric nomenclature should follow the rules contained in *Official Methods of Analysis*, "Definitions of Terms and Explanatory Notes.");

Don't Forget!

the

96th AOAC

Annual International Meeting

is at a new location

THE SHOREHAM HOTEL

Washington, DC

October 25-28, 1982

In This Issue