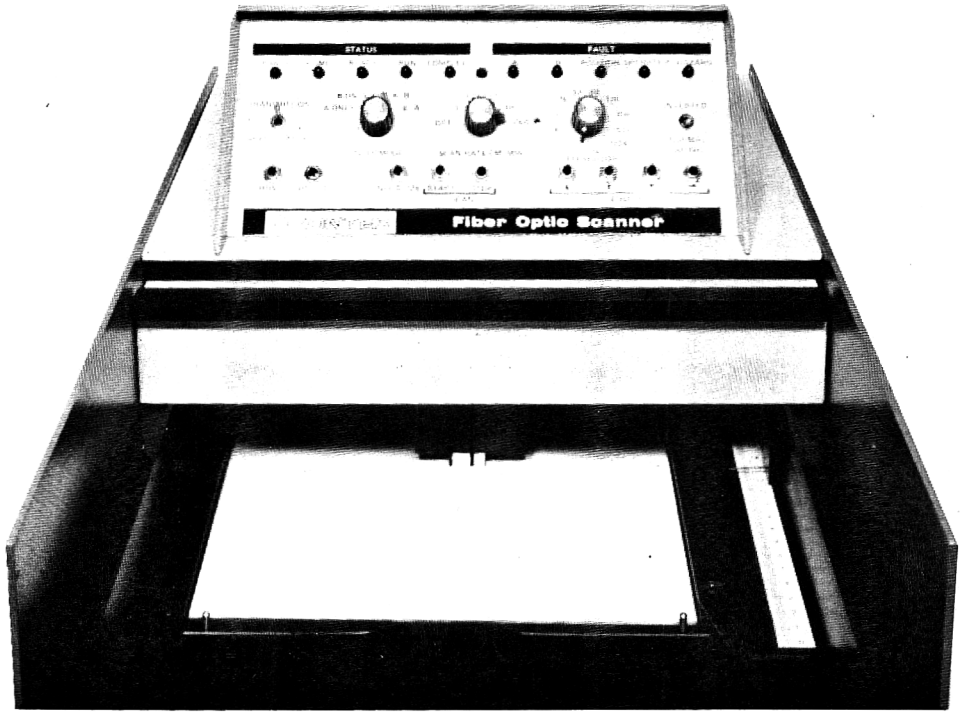
ISSN 0004-5756

A O A C

NOVEMBER 1982
VOL. 65, NO. 6

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS JOURNAL

JANCA2 65(6) 1297–1565 (1982)



Scanning Cellulose Acetate? TLC Plates? Gels?

Our new multi-media, patented[1] fiber optic scanner is designed to detect the optical properties of a wide variety of media. Applications are virtually unlimited.

Scannable media includes autoradiograms, TLC plates, paper, glass, textiles, metals, gels, powders, films and others. Qualitative internal and external strain analysis scans are also possible using dual polarizing filters.

A wide range of operating modes, scan head configurations and spectral parameters provide a unique combination of sensitivity and flexibility. Spectrally & thermally stabilized detectors and electronics provide an overall reproducibility of ± 1% or better.

We could go on about our patented optics, compact size, ease of operation, low power consumption, and more —and we do in our scanner brochure with detailed specifications.

[1]Pat. No. 3,562,539 - 3,924,948

KONTES

You Can't Buy Better Laboratory Products.™

Vineland, NJ 08360 (609) 692-8500

Exclusive Distributors: **KONTES OF ILLINOIS,** Evanston, Illinois

KONTES OF CALIFORNIA, San Leandro, California

TrAC

trends in analytical chemistry

For the specialist and non-specialist working with analytical methods, our new monthly journal — TrAC — is essential reading.

Easy to read...

no jargon, no highly-specialised terminology

Informative yet informal...

brings you not only purely scientific reviews, but also regular information about the broader implications of analytical chemistry — both practical and theoretical aspects — plus topical news items from all over the world.

Affordable...

if you need to keep up with current trends and future perspectives in analytical chemistry, you can't afford to miss TrAC

Look at what you've missed if you haven't seen TrAC

- GC-MS in diagnosis of human metabolic diseases, *by E. Jellum.*
- Clinical analysis of lipids using microbial enzymes, *by T. Uwakima and O. Terada*
- Analytical chemistry as an information science, *by B. Kowalski*
- Fluorescence methods for studying speciation of pollutants in water, *by W. Rudolf Seitz*
- Characterization of individual airborne particles - methods used and their limitations, *by M. Grasserbauer and C. Malami*
- The limitations of radioimmunoassay, *by R. Yalow*
- Solid surface sample handling techniques in organic trace analysis, *by R.W. Frei and V.A. Th. Brinkman*
- The Analytical Division of IUPAC, *by T.S. West*

... and many more

TrAC

trends in analytical chemistry

Don't despair - subscribe now! and keep up-to-date with what others are doing in the fields of

- analytical chemistry
- clinical and biochemistry
- toxicology
- environmental research
- geology

If you're still not convinced, ask for a free sample copy of

TrAC

Send to:

ELSEVIER

ACT NOW!

Please enter my subscription to the:

☐ Personal Monthly Edition (12 issues) from _____ (month). *(Personal subscriptions must be accompanied by a cheque or payment advice).* US $42.50; UK £20.00; Europe 91.50 Dutch guilders; Japan Yen 12400; elsewhere 95.50 Dutch guilders

☐ Library Edition Vol. 1, 1981/82 (16 issues plus compendium volume containing all review articles and indexed for easy retrieval) and send invoice for U.S $104.00/260 Dutch guilders

☐ Please send a free sample copy of TrAC

Name _____

Address _____

_____ Zip/Post Code _____

P.O. Box 330,
1000 AH Amsterdam,
The Netherlands
Tel.: (20) 5803-450

52 Vanderbilt Ave.,
New York, NY 10017,
U.S.A.
Tel.: (212) 867-9040

7233

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.

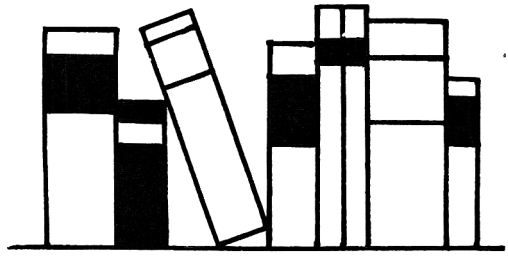
As analytical needs change and as knowledge and techniques advance, new and revised methods are validated by AOAC. These are incorporated into annual updates which are included in the purchase price.

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

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 NOVEMBER 1982 No. 6

CONTENTS

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

MAGRUDER
FERTILIZER CHECK SAMPLE PROGRAM

Quality Assurance

Interlaboratory evaluation of methods of analysis and personal performance is increasingly important. Instrumentation in laboratories is a great time saver but needs careful control. Profit margins in the fertilizer industry continue to diminish, calling for close quality assurance. These areas can be improved by an interlaboratory statistical evaluation of your laboratory, which the Magruder Check Sample Program offers to all organizations involved in fertilizer analysis.

Program

Each month's sample(s) contain the primary plant nutrients N, P, and K; and about four samples each year contain secondary and micronutrients. Samples are analyzed by methods chosen by the individual laboratories. A comprehensive statistical summary is prepared, noting interlaboratory bias, precision, and ranking of coded individual laboratories. Each subscriber receives this summary and his confidential ranking to evaluate his performance.

Participants

There are about 140 laboratories that subscribe to the Magruder program. All of the state control laboratories participate, and the remaining number is about equally divided between U.S. industrial and foreign laboratories. The annual subscription fee is $75.00.

Subcription Information

Applications may be obtained by writing:

Mr. Howard P. Moore
Feeds and Fertilizers
Department of Agriculture
Reynoldsburg, Ohio 43068

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.

NOTICE TO READERS ABOUT COPYING

PESTICIDE RESIDUES

Selection of Enzyme Sources for Improved Sensitivity in Enzymatic Determination of Organophosphorus Pesticides

SANA UDAYA BHASKAR[1] and NANGUNERI V. NANDA KUMAR
Sri Venkateswara University, Department of Zoology, Tirupati-517 502, India

The Michaelis-Menten constant (K_m) for cholinesterases from different liver sources, the inhibitor concentration for 50% inhibition (I_{50}), and the lower limit of estimation were determined for 3 organophosphorus pesticides and their oxygen analogs. Maximum sensitivity in organophosphorus pesticide determinations can be achieved by choosing the enzyme source which has a higher K_m value. An inverse relationship is suggested between K_m and the lower limit of estimation.

Enzymatic methods based on the cholinesterase (ChE) inhibition technique have been combined with colorimetry (1-4) and thin layer chromatography (5-8) to detect and determine organophosphorus pesticides. The lower limits of estimation and detection vary with different enzyme sources (9-11). Little has been published on the causes of the variation of sensitivity to organophosphorus compounds. We have attempted to draw a relationship between the Michaelis-Menten constant (K_m), an index of enzyme-substrate affinity, and I_{50}, the inhibitor concentration for 50% inhibition to understand this variation. Our procedure enables the selection of ChE with higher sensitivity for estimating trace amounts of organophosphorus pesticide residues.

Experimental

All chemicals were analytical grade. Fenitrothion 95% and dimethoate 97% were obtained from Rallies India Ltd, Bombay; methyl parathion 95% was obtained from Ciba-Geigy Ltd, Basel, Switzerland. The oxygen analogs fenitrooxon, omethoate, and methyl paraoxon were prepared as described earlier (1).

ChE from fresh livers of sheep and rat, and liver acetone powders of pig and horse were assayed by the method reported earlier (1-3); K_m values were determined graphically (12). The concentration of organophosphorus inhibitors (fenitrothion, fenitrooxon, methyl parathion, methyl paraoxon, dimethoate, and omethoate) required for 50% inhibition (I_{50}) and the lower limit of estimation of the organophosphorus pesticides were deduced from the calibration curves for percentage ChE inhibition vs pesticide concentration (1-4).

Results and Discussion

From the experimental results, Michaelis-Menten constant and I_{50} values showed an inverse relationship (Table 1). Enzymes with higher K_m values had lower I_{50} values and limits of estimation (higher sensitivity). Similar results were observed for all organophosphorus pesticides tested. Sheep and pig liver ChE, with relatively high K_m values, required relatively less organophosphorus pesticide to cause 50% ChE inhibition. These enzyme sources also showed higher sensitivity in determination of organophosphorus pesticide. Rat and horse liver enzymes, with low K_m values, required relatively higher amounts (2 to 10 times) of inhibitor to cause 50% ChE inhibition, and the lower limit of estimation was relatively higher. From these observations, a relationship between K_m and the lower limit of estimation can be suggested, based on the enzyme-substrate affinity. It is well known that the organophosphorus compounds and the substrate 1-naphthyl acetate are the esters competing for the same active site of the enzyme. Low (high K_m) or high (low K_m) affinity of enzyme towards substrate seems to affect the binding capacity of the enzyme. For a lower K_m value, the substrate would overcome the inhibition of ChE by the organophosphorus compound. For a higher K_m value of ChE, the inhibitor would overcome the substrate. This relationship can be predicted by considering the appropriate kinetics. However, ChE inhibition by organophosphorus pesticides is irreversible (13). Although the kinetics describe reversible conditions only, at low concentrations of inhibitor and high concentrations of ChE, the approximations may be valid.

From Equation VIII.6 (ref. 13, all symbols with usual meaning):

[1] Present address: National Institute of Oceanography, Biological Oceanography Division, Dona Paula, 403004, Goa, India.

Received January 5, 1982. Accepted March 23, 1982.

Table 1. Relationship between K_m of ChE, I_{50} values, and lower limit of estimation of organophosphorus pesticides [a] for various enzyme sources

Enzyme source	K_m (mM 1-naphthyl acetate)	Lower limit of estimation, μg/mL					
		Fenitrothion	Fenitrooxon	Methyl parathion	Methyl paraoxon	Dimethoate	Omethoate
Pig liver [b]	0.26	1.00 (2.93)	0.05 (0.23)	0.80 (2.59)	0.03 (0.05)	1.00 (3.62)	0.29 (0.29)
Sheep liver	0.22	1.00 (2.56)	0.05 (0.25)	1.00 (3.50)	0.05 (0.26)	1.00 (3.59)	0.10 (0.75)
Rat liver	0.18	3.00 (5.54)	0.10 (0.38)	5.00 (13.0)	0.30 (1.02)	7.00 (24.2)	0.50 (1.99)
Horse liver [b]	0.14	10.00 (28.9)	1.00 (2.41)	12.00 (41.6)	0.80 (2.63)	20.00 (65.7)	1.50 (6.71)

[a] Values are mean of 6 values, except lower limit of estimation. I_{50} values ($\times 10^{-8}$M) are given in parentheses.
[b] Acetone powder.

$$v = V \text{ max.}/1 + (K_m/S) \times (1 + i/K_i)$$

at I_{50}

$$v = V \text{ max.}/2 \text{ (approx.)}$$

Then

$$(1 + K_m/S) \times (1 + I_{50}/K_i) \simeq 2$$

$$(K_m/S) \times (1 + I_{50}/K_i) \simeq 1$$

$$1 + (I_{50}/K_i) \simeq S/K_m$$

So it can be predicted that I_{50} varies inversely with K_m, which is also observed from the experimental results. Based on the above observations, tested with 6 organophosphorus pesticides, the authors recommend that the ChE source with higher K_m is the more advantageous ChE source for estimating trace amounts of organophosphorus pesticides.

Acknowledgment

S. Udaya Bhaskar gratefully acknowledges CSIR, New Delhi, for financial assistance.

References

(1) Udaya Bhaskar, S., & Nanda Kumar, N. V. (1980) *Talanta* **27**, 757–758
(2) Nanda Kumar, N. V., & Udaya Bhaskar, S. (1980) *J. Food Sci. Technol.* **17**, 153–155
(3) Nanda Kumar, N. V., & Udaya Bhaskar, S. (1980) *Indian Food Packer* **34**(5), 9–11
(4) Nanda Kumar, N. V., & Ramasundari, M. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 536–538
(5) Nanda Kumar, N. V., Visweswaraiah, K., & Majumdar, S. K. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 641–643
(6) Nanda Kumar, N. V. Visweswaraiah, K., & Majumdar, S. K. (1976) *Agric. Biol. Chem.* **41**, 431–432
(7) Udaya Bhaskar, S., & Nanda Kumar, N. V. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 1312–1314
(8) Udaya Bhaskar, S. (1982) *Talanta* (in press)
(9) Mendoza, C. E. (1977) *Residue Rev.* **43**, 105–142
(10) Mendoza, C. E. (1974) *Residue Rev.*, **50**, 43–72
(11) Udaya Bhaskar, S. (1981) Ph.D. thesis, Sri Venkateswara University, Tirupati, India, 101 pp.
(12) Line Weaver, H., & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–661
(13) Dixon, M., & Webb, E. C. (1979) *Enzymes*, Longman Group, London, UK, 1116 pp.

Improved Method for High Pressure Liquid Chromatographic Determination of Chlorophacinone in Mouse Tissue

JOSEPH B. ADDISON
Governors State University, College of Arts and Sciences, Division of Science, Park Forest South, IL 60466

A simplified method is described for the determination of chlorophacinone, 2-[(*p*-chlorophenyl)-phenylacetyl]-1,3-indandione, in homogenized mice. Chlorophacinone is extracted with acetonitrile. After Florisil cleanup, the extract is injected into a high pressure liquid chromatograph for reverse phase chromatography on a polar Lichrosorb NH_2 (10 μm) column, with a mobile phase of acetonitrile-water (80 + 20). An injection containing 70 ng chlorophacinone produces ½ scale peaks at 254 nm with a full scale absorbance of 0.1 unit, an order of magnitude improvement over the sensitivity reported earlier with a 280 nm detector. Six homogenized mice samples and six spiked homogenized mice samples were quantitatively analyzed for trace levels of chlorophacinone by this method. Recoveries from spiked samples, as determined by peak height measurement, were >95%. Mean retention time for the chlorophacinone peaks in all samples was 6.05 ± 0.05 min. Chlorophacinone levels determined in homogenized whole mouse samples ranged from 0 to 63 ppm.

Chlorophacinone was introduced as a rodenticide about 20 years ago. An LD_{50} of 20.5 mg/kg has been reported in mice and rats (1).

Several attempts have been made to develop a sensitive, rapid, direct determination of chlorophacinone in animal tissue. A spectrophotometric method (2) proved to be very insensitive (100 mg). Trace level determinations of 10–20 ppb chlorophacinone in biological samples (3, 4) involved indirect oxidation and bromination with subsequent electron capture detection. Such derivatizations prior to gas chromatography result in low recoveries, especially when large amounts of interfering compounds are present. A thin layer chromatographic method has been reported (5) and is the basis of the most recent determination of chlorophacinone by high pressure liquid chromatography (HPLC) (6). This reverse phase ion-pair chromatographic method offers a direct determination of chlorophacinone in less complex matrixes in which there is little or no interference from other compounds. Column deterioration occurs because, in time, the counter-ion reacts with the octadecyl C_{18} column packing and chlorophacinone analysis becomes impossible.

Received May 13, 1981. Accepted April 1, 1982.

Experimental

Reagents and Apparatus

All solvents were HPLC grade from Caledon Laboratories Ltd, Georgetown, Ontario, Canada L7G 4R9.

(a) *Chlorophacinone standard.* 2-[(*p*-Chlorophenyl)phenylacetyl]-1,3-indandione (Chempar Chemical Co., Inc., New York, NY) was used without further purification. Standard solutions were prepared by dissolving known concentrations in acetonitrile.

(b) *Liquid chromatograph.*—Varian 5000 LC, equipped with 2 mm × 25 cm Lichrosorb NH_2 (10 μm) column and a fixed 254 nm UV detector. Operating conditions: column temperature, ambient; detector sensitivity, 0.002 absorbance unit full scale; mobile phase, acetonitrile–water (80 + 20) at flow rate of 1 mL/min. Pump mobile phase through column at 22 atm.

Sample Preparation and Extraction

Kill mice by placing swab of cotton soaked in ether in cage for ca ½ h. Homogenize each mouse (average weight, 13.5 g) in Polytron (Type PT 20 OD, Stanton-Thompson Ltd, London, UK), and place homogenized tissue in aluminum foil container. Add, by pipet, a known amount of chlorophacinone standard solution and thoroughly blend solution with tissue. Freeze-dry spiked sample by using Virtis automatically refrigerated freeze-dryer, and store in freezer until ready for analysis.

To extract chlorophacinone for HPLC analysis, blend whole freeze-dried sample with ca 200 mL acetonitrile ca 30 min at medium speed on multi-wrist shaker equipped with electronic timer. Filter mixture and transfer filtrate to separatory funnel containing 100 mL hexane. Shake vigorously ca 5 min, and then let stand until the 2 liquid layers are separated. Drain lower acetonitrile layer and concentrate that layer to ca 5 mL on rotary evaporator. Use entire 5 mL concentrate for Florisil cleanup.

0004-5756/82/6506-1299-03$01.00

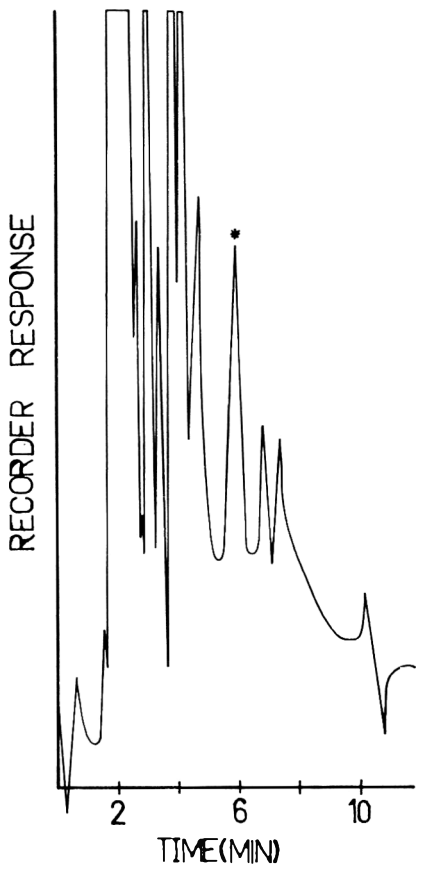


Figure 1. Typical chromatogram of spiked mouse extract (* chlorophacinone peak).

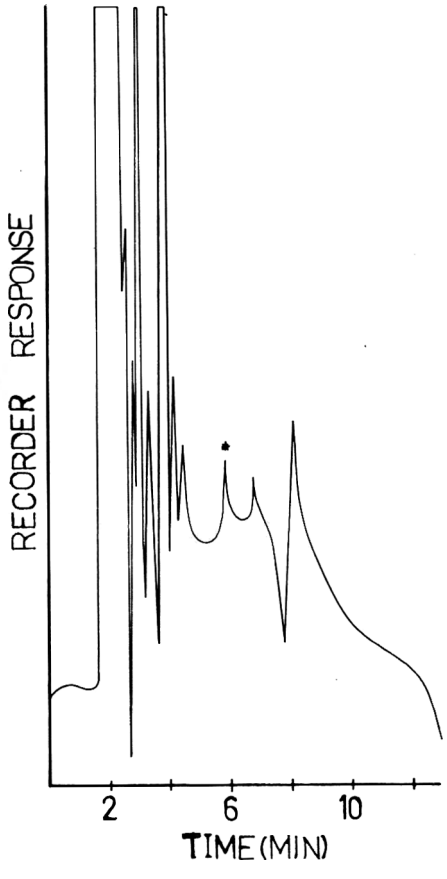


Figure 2. Typical chromatogram of homogenized mouse sample extract (* chlorophacinone peak).

Column Cleanup

Place 2 in. Florisil (60–100 mesh) in 2 cm id glass buret with sintered glass bottom. Gently tap column to settle Florisil. Add 1 in. anhydrous Na_2SO_4 on top of Florisil, and wash column with 50 mL dichloromethane. Pipet the 5 mL extract onto column and wash column with 50 mL dichloromethane, followed by 30 mL acetonitrile. Discard wash, and elute chlorophacinone with 100 mL methanol. Evaporate eluate in rotary evaporator to ca 1 mL for direct HPLC analysis.

Calculations

Calculate concentration of chlorophacinone in sample as follows:

$$\text{Chlorophacinone, ppm} = 10^3 \times [H_t\,(\text{sample})/H_t\,(\text{std})] \times [\text{ppm (std)}/V\,(\text{sample})] \times [V\,(\text{final})/V\,(\text{inj.})]$$

where H_t (sample) = peak height of chlorophacinone sample; H_t (std) = peak height of chlorophacinone standard solution; ppm (std) = concentration of chlorophacinone corresponding to H_t dried homogenized mouse; V (final) = final volume (mL) of acetonitrile extract; V (inj.) = volume (μL) of extract corresponding to H_t (sample).

Results and Discussion

Previous work has been reported (6) on HPLC determination of chlorophacinone by a reverse phase ion-pair chromatography technique. In this work, the alkaline solvent system produced sharp and polarity response peaks on a microparticulate reverse phase column, Varian Micropak MCH 10. HPLC chromatograms also showed a reversed order of elution, with chlorophacinone eluting ahead of the solvent front. Retention times did not respond to changes in the mobile phase composition, and prolonged

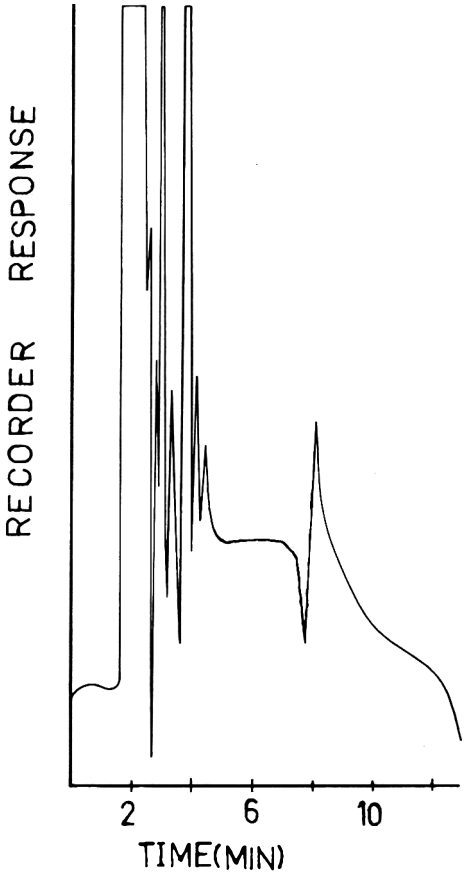


Figure 3. Typical blank mouse extracts.

use of primary, secondary, and tertiary amine counter-ions caused deterioration of the Micropak column. Also, in the presence of other interfering compounds such as lipid residue, chlorophacinone could not be satisfactorily separated by ion-pair chromatography. A polar Lichrosorb NH_2 (10 μm) column (2 mm × 24 cm), employed in the reverse phase mode, offered the best HPLC method for determining chlorophacinone in the presence of other polar-eluting compounds in the sample extracts. The mobile phase was acetonitrile–water (80 + 20) instead of the more complicated ion-pair chromatographic method, which involves the addition of counter-ions to the mobile phase. Figures 1, 2, and 3 show typical chromatograms of spiked extract, homogenized sample extract, and blank extract, respectively. A plot of peak height vs concentration of spiked chlorophacinone was linear over the concentration range 0–80 ng per injection; above 80 ng, there was a sharp departure from linearity. The linear correlation coefficient of the plot was estimated to be 98%.

Recovery from each of 6 spiked homogenized samples was >95% by peak height. Mean retention time for chlorophacinone in all samples was 6.05 ± 0.005 min. Blank samples showed that other polar-eluting compounds were present after Florisil cleanup; however, these peaks did not appear in the region where chlorophacinone elutes. The method was therefore successfully used to determine concentrations of chlorophacinone in animal tissues and especially in the presence of other materials, which were otherwise impossible to remove when the whole mouse is homogenized for chlorophacinone estimation. The lower limit of detectability in animal tissue extracts by this method was estimated to be 4 ppb for 1 μL injection. Twenty ng standard chlorophacinone solution produced 61% of full scale peaks at 254 nm with a full scale absorbance of 0.002 unit.

REFERENCES

(1) Rowe, F. P., & Redfern, R. (1968) *Ann. Appl. Biol.* **61**, 322–326
(2) *Technical Report on Chlorophacinone* (1970) Chempar Chemical Co., 260 Madison Ave, New York, NY
(3) Bullard, R. W., Holguin, G., & Peterson, J. E. (1975) *J. Agric. Food Chem.* **23**, 72–74
(4) Young, R. W., Parry, O. D., & Dickinson, J. K. (1975) *Bull. Water Resources Research Center* **90**, 1–20
(5) *Report on Improved Technique for the Identification of Chlorophacinone* (1972) Lyonaaise Industrielle Pharmacutique, B.P. No. 106, Lyon R.P. 69, Lyon (3e), France
(6) Grant, R. G., & Pike, R. K. (1979) *J. Assoc. Off. Anal. Chem.* 62, 1001–1003

Simultaneous Liquid Chromatographic Determination of Thiabendazole, o-Phenylphenol, and Diphenyl Residues in Citrus Fruits, Without Prior Cleanup

YOSHIMI KITADA, MICHIKO SASAKI, and KAORU TANIGAWA
Nara Prefectural Institute of Public Health, 57-6 Ohmori, Nara 630, Japan

A simple, rapid, efficient method has been developed for determining thiabendazole, o-phenylphenol, and diphenyl in citrus fruits by using high performance liquid chromatography, with fluorescence or ultraviolet detection. The compounds are extracted with ethyl acetate and separated from soluble fruit components on a LiChrosorb RP-8 column. Recovery of these compounds added to citrus fruits at 5 or 50 ppm levels was >93%; the limit of detection for the compounds is 1 ppm.

Thiabendazole (TBZ), *o*-phenylphenol (OPP), and diphenyl (DP) are widely used as fungistats on citrus fruit to protect it from decay during storage and transport. In Japan, these compounds have been registered as food additives. The established tolerances in citrus fruit are 10 μg/g for TBZ and OPP, and 70 μg/g for DP.

High performance liquid chromatography (HPLC) has been shown to be useful for determining TBZ (1, 2), OPP (3–6), and DP (7). Detection of TBZ is mainly based on its fluorescence, and for OPP and DP, on their strong ultraviolet (UV) absorption. However, we used the fluorescence characteristic of OPP. Recently, a simultaneous gas-liquid chromatography (GLC) determination of TBZ, OPP, and DP was reported (8). Few methods have been published for HPLC analysis of these compounds.

The proposed method for determining TBZ, OPP, and DP in citrus fruit involves extraction with ethyl acetate, and concentration. The determination by HPLC without prior cleanup is simple, accurate, and time-saving.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Shimadzu Model LC-2F (Shimadzu Seisakusho Ltd, Kyoto, Japan).

(b) *Column.*—Stainless steel, 4 mm id × 25 cm, with LiChrosorb RP-8, 7 μm (E. Merck, Darmstadt, GFR).

(c) *Detector.*—Shimadzu Model RF-510LC fluorescence spectrometer equipped with xenon lamp, and Shimadzu Model UVD-2 monitor (Shimadzu Ltd).

(d) *Mixer.*—Matsushita Model MK-1010 (Matsushita Electric Industry Ltd, Osaka, Japan).

(e) *High-speed blender.*—Nihon Seiki Model AM-7 equipped with stainless steel cup (Nihon Seiki Ltd, Tokyo, Japan).

(f) *Rotary vacuum evaporator.*—Sibata Model S (Sibata Chemical Apparatus Mfg. Ltd, Tokyo, Japan).

(g) *Sample filtering system.*—25 mm filter holder and Fluoropore filters, 1.0 μm pore size (Sumitomo Electric Industry Ltd, Osaka, Japan).

(h) *Gas-liquid chromatograph.*—Shimadzu Model GC-5A (Shimadzu Ltd) equipped with dual 3 mm id × 2 m glass columns (5% polyethylene glycol 20M on 60–80 mesh Chromosorb W) attached to flame ionization detectors. Gas flow rates: nitrogen 40, hydrogen 45, air 800 mL/min.

Reagents

(a) *Solvents.*—Methanol and ethyl acetate (analytical grade, Wako Pure Chemical Industries Ltd, Osaka, Japan).

(b) *Standard solution.*—100 μg TBZ, OPP, and DP/mL. Dissolve 0.100 g thiabendazole (Tokyo Kasei Industry Ltd, Tokyo, Japan), *o*-phenylphenol, and diphenyl (Wako) in 1 L methanol.

(c) *Mobile phase.*—Methanol—pH 8.0 phosphate buffer (60 + 40). Dissolve 3.265 g Na_2H-$PO_4.12H_2O$ and 0.120 g KH_2PO_4 in water. Dilute to 1 L. Mix 600 mL methanol and 400 mL phosphate buffer.

Preparation of Sample Extract

Use 3 different kinds of fruit, i.e., lemon, orange, and grapefruit. For each, slice 3–5 fruit and homogenize with mixer. Weigh 10 g mixture into high-speed blender cup and add 40 mL ethyl acetate. Extract by homogenizing 3 min at 10 000 rpm, then suction-filter supernate through paper (Tokyo filter paper No. 5C, Tokyo, Japan) into 200 mL round-bottom 24/40 ⅁ flask. Homogenize precipitate with an additional 40 mL ethyl acetate, and then filter. Wash residue

Received December 30, 1981. Accepted April 1, 1982.

0004-5756/82/6506-1302-0301.00

Table 1. Recovery of added thiabendazole (TBZ), o-phenylphenol (OPP), and diphenyl (DP) from citrus fruits [a]

Fruit	Added, μg/g	TBZ			OPP			DP		
		Rec., %	SD	CV, %	Rec., %	SD	CV, %	Rec., %	SD	CV, %
Lemon	5	95.2	2.0	2.12	98.9	0.9	0.93	94.3	5.5	5.82
	50	94.0	1.9	2.05	95.6	1.3	1.33	95.9	2.4	2.51
Orange	5	93.5	2.0	2.16	93.8	1.9	2.05	96.5	4.1	4.29
	50	95.1	1.1	1.18	96.4	0.2	0.25	94.6	0.7	0.70
Grapefruit	5	98.6	3.1	3.11	99.6	2.3	2.30	95.0	5.1	5.35
	50	96.9	2.0	2.09	94.1	1.9	2.06	96.9	2.0	2.09

[a] Results of 6 trials.

on filter paper with 30 mL ethyl acetate. Concentrate combined ethyl acetate extracts to ca 2 mL on rotary vacuum evaporator (15 mm Hg, 40°C water bath). Transfer concentrate to 10 mL volumetric tube with methanol, mix, and then filter through 1.0 μm filter.

High Performance Liquid Chromatography

Dilute standard solution with methanol to prepare solutions containing 5, 10, 20, 30, 40, and 50 μg TBZ, OPP, and DP/mL. Use the following conditions: excitation wavelength of fluorescence spectrophotometer 285 nm, and emission 350 nm (range, 16; sensitivity control, 8); wavelength of UV monitor 254 nm (0.16 AUFS); mobile phase flow rate 1.0 mL/min (isocratic run); column temperature 55°C. Inject 10 μL of each standard solution into chromatograph, and prepare each calibration curve by plotting peak heights of TBZ, OPP, and DP against each concentration (μg/mL). Chromatograph sample extract under same conditions. Read each amount (μg/mL) of TBZ, OPP, and DP in extract. These amounts are the concentration (μg/mL) of each chemical in sample fruit.

Results and Discussion

Table 1 shows recoveries from citrus fruits fortified with 5 or 50 ppm TBZ, OPP, and DP, after homogenization with the mixer. A plot of chromatographic peak height vs concentration showed linear correlation from 5 to 50 ppm. Figure 1 shows peak separation for the 3 compounds. TBZ and OPP show small peaks when UV detection is used (Figure 1B); DP appears as a small peak with fluorometric detection. The other peaks in the chromatograms are due to the grapefruit extract.

Because TBZ, OPP, and DP were to be extracted with organic solvent, we compared the efficiency of dichloromethane, methanol, and ethyl acetate for extracting the compounds from citrus fruit. Recoveries were satisfactory only with ethyl acetate. Recoveries of DP with dichloromethane and methanol were 73 and 30%, respectively.

Isshiki et al. (8) reported that it was necessary to adjust the pH to 7–10 to extract TBZ, OPP, and DP with ethyl acetate from phosphate buffer. The extraction rate of TBZ decreased under acidic conditions, while TBZ and OPP decreased under more basic conditions. The extraction rate for TBZ was 80% at pH 3.5 in their investigation. Therefore, the extractability of TBZ, OPP, and DP

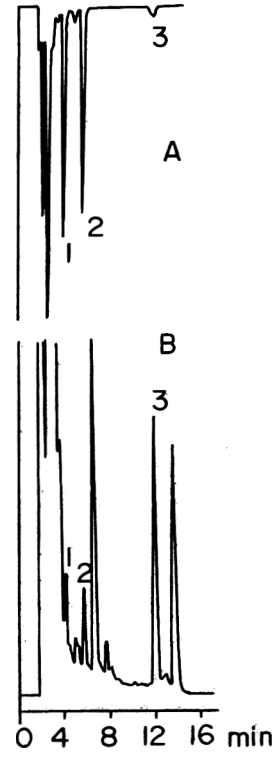


Figure 1. Liquid chromatogram of grapefruit extract (1 g grapefruit/mL) containing: 1, 10 ppm thiabendazole; 2, 10 ppm o-phenylphenol; 3, 21 ppm diphenyl A, fluorometric; B, UV.

was compared by adding Na_2HPO_4 to sample fruit. Recoveries were satisfactory with or without added Na_2HPO_4, so it was not used.

To check the background interference from natural substances on TBZ and OPP, untreated fruits were analyzed. No peak corresponding to TBZ or OPP was present in the chromatograms. For DP, HPLC and GLC analyses were compared for 6 fruit samples (9). The correlation coefficient between HPLC and GLC analyses was 0.988.

A UV monitor was employed for DP and a fluorescence spectrophotometer for TBZ and OPP. Fluorometry was more sensitive and specific than spectrometry for TBZ and OPP, and therefore, it decreased the influence of natural fruit components. Fluorescence intensity was strongest when excitation and emission wavelengths were set at 305 and 350 nm (TBZ) and 285 and 345 nm (OPP), respectively. We set excitation and emission wavelengths at 285 and 350 nm, respectively.

The detection limit for the method was 1 ppm TBZ, OPP, and DP. The entire procedure can be completed in 40 min/sample.

Acknowledgments

The authors express their appreciation to T. Itano of this institute for encouragement.

References

(1) Maeda, M., & Tsuji, A. (1976) *J. Chromatogr.* **120**, 449–455
(2) Isshiki, K., Tsumura, S., & Watanabe, T. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 747–749
(3) Reeder, S. K. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 162–164
(4) Hayashi, T., et al. (1979) *Eisei Kagaku* **25**, 131–135
(5) Ott, D. E. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 1465–1468
(6) Aitzetmuller, K., & Arzberger, E. (1981) *J. High Resolut. Chromatogr. Chromatogr. Commun.* **4**, 242–243
(7) Reeder, S. K. (1975) *J. Assoc. Off. Anal. Chem.* **58**, 1013–1014
(8) Isshiki, K., Tsumura, S., & Watanabe, T. (1980) *Nippon Nogeikagaku Kaishi* **54**, 1045–1050
(9) Ito, Y. (1977) *Eisei Kagaku* **23**, 129–132

COLOR ADDITIVES

Ion-Pair High Performance Liquid Chromatographic Separation and Detection of Subsidiary Dyes in Synthetic Food Colors

FRANK E. LANCASTER and JAMES F. LAWRENCE
Health and Welfare Canada, Food Research Division, Food Directorate, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario, Canada K1A OL2

A high performance liquid chromatographic method utilizing a reverse phase ion-pair technique is presented for separating subsidiary dyes from commercial samples of Amaranth, Sunset Yellow FCF, and Tartrazine. After separation, the subsidiaries or primary dyes are quantitated by using known standards. Recoveries of subsidiaries, 1-(4-sulfo-1-naphthylazo)-2-naphthol-6-sulfonic acid, disodium salt from Amaranth; 1-*p*-sulfophenylazo-2-naphthol-3,6-disulfonic acid, trisodium salt from Sunset Yellow FCF; 3-carboxy-5-hydroxy-1-*p*-sulfophenyl-4-phenylazo-pyrazole, disodium salt from Tartrazine, ranged from 92 to 102% at the spiking levels studied (0.06-16%, depending on the subsidiary). The method was successfully applied to the analysis of commercial samples.

Amaranth, Sunset Yellow FCF, and Tartrazine are 3 of the synthetic colors permitted for use in foods in Canada (1). Because they are regulated substances, limits are placed on the levels of impurities permitted in these additives, and each batch of dye is chemically analyzed to ensure compliance.

Amaranth (formerly FD&C Red No. 2), the trisodium salt of 1-(4-sulfo-1-naphthylazo)-2-naphthol-3, 6-disulfonic acid, is prepared by coupling 2-naphthol-3,6- disulfonic acid, sodium salt (R-salt) with diazotized naphthionic acid (Figure 1). If any other sulfonic acids such as 2-naphthol-6-sulfonic acid are present in the R-salt, they will also couple with the diazotized amine and produce subsidiary colors. Examination of commercial samples of Amaranth has shown that the most frequent and abundant subsidiary dye does result from 2-naphthol-6-sulfonic acid and is the disodium salt of 1-(4-sulfo-1-naphthylazo)-2-naphthol-6-sulfonic acid (commonly referred to as Fast Red E).

Sunset Yellow FCF (FD&C Yellow No. 6), the disodium salt of 1-*p*-sulfophenylazo-2-naphthol-6-sulfonic acid is prepared by coupling diazotized sulfanilic acid with 2-naphthol-6-sulfonic acid, sodium salt (Schaeffer's salt) (Figure 2). The main, lower-sulfonated dyes of Sunset Yellow FCF are due to 2-naphthol in the Schaeffer's salt, or aniline in the sulfanilic acid. Commercial samples of Sunset Yellow FCF usually contain the lower-sulfonated dye formed from the coupling of 2-naphthol and diazotized sulfanilic acid. Higher-sulfonated subsidiaries can result when the diazotized sulfanilic acid is coupled with impurities in the Schaeffer's salt, such as 2-naphthol- 6,8-disulfonic acid, sodium salt (G-salt), or, more likely, R-salt. The latter subsidiary is added to Sunset Yellow FCF by some manufacturers, in an amount not exceeding allowable limits, to inhibit precipitation of the calcium salt when orange-emulsion concentrates containing gum arabic are prepared.

Tartrazine (FD&C Yellow No. 5), the trisodium salt of 3-carboxy-5-hydroxy-1-*p*-sulfophenyl-4-*p*-sulfophenylazopyrazole, is prepared by coupling diazotized sulfanilic acid with 3-carboxy-1-(4-sulfophenyl)-5-hydroxypyrazolone (Figure 3). The most likely lower-sulfonated dyes of Tartrazine are due to aniline in the sulfanilic acid or 1-phenyl-3-carboxy-5-pyrazolone present in the other intermediate. Dolinsky reports (2) that the most abundant subsidiary dye usually present in Tartrazine occurs as a result of aniline in the sulfanilic acid, which produces 3-carboxy-5-hydroxy-1-*p*-sulfophenyl-4-phenylazo-pyrazole, disodium salt during the synthesis.

In the last 3 years, over 90% of the primary colors certified by, or to, the Health Protection Branch were Amaranth, Sunset Yellow FCF, or Tartrazine. Consequently, these 3 dyes were selected for this study.

Subsidiary dyes may be separated from primary dyes using thin layer chromatography (TLC) (2-6), column chromatography (7, 8), or a combination of the two (9, 10). Paper chromatography (11) or amyl alcohol extraction (12) may also be utilized. However, these methods are often laborious, time-consuming, or do not pro-

Received January 15,1982. Accepted March 23,1982.

0004-5756/82/6506-1305-06$01.00

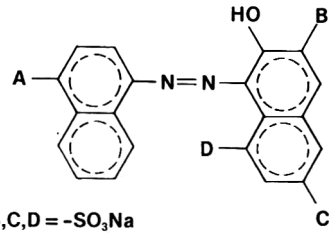


Figure 1. Amaranth, sulfonated at A + B + C. Higher sulfonated subsidiary sulfonated at A + B + C + D; lower sulfonated subsidiary (Fast Red E) sulfonated at A + C.

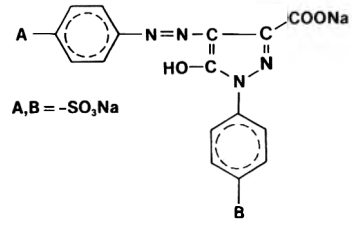


Figure 3. Tartrazine, sulfonated at A + B. Lower sulfonated subsidiaries sulfonated at A or B (selected for analysis).

duce a satisfactory separation of the compounds of interest.

In recent years, high performance liquid chromatography (HPLC) has become a popular technique for the analysis of synthetic food colors and their contaminants. Ion exchange (10, 13) and reverse phase (14) HPLC have been used for determining intermediates and subsidiaries, and reverse phase ion-pair HPLC (15–20) has been found particularly useful for the separation and detection of a large number of food colors.

We required a method that was rapid, accurate, and simple for the determination of the major subsidiary dyes in Amaranth, Sunset Yellow FCF, and Tartrazine. HPLC appeared to be the logical approach. Because current ion-exchange HPLC methods (21, 22) require lengthy equilibration procedures in addition to gradient elution for the separations, ion-pair reverse phase HPLC in the isocratic mode was initially selected. The method developed meets and surpasses the necessary criteria. Additional minor subsidiaries are resolved during analysis and the method is amenable to automation. Analyses require 8–14 min and results are very reproducible. By using known standards, the primaries can also be quantitated.

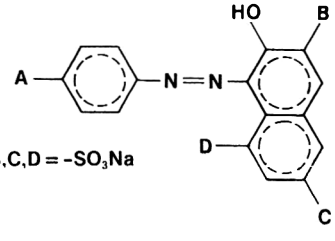


Figure 2. Sunset Yellow FCF, sulfonated at A + C. Higher sulfonated subsidiaries sulfonated at A + B + C (selected for analysis) or A + C + D; lower sulfonated subsidiaries sulfonated at A or C.

Experimental

Apparatus and Reagents

(a) *Liquid chromatograph.*—Waters Associates (Milford, MA) Model 6000A pump, Model 450 variable wavelength detector, and Rheodyne 7125 syringe-loading injection port with 20 μL loop. Operating conditions: flow rate, 1.0 mL/min; detector sensitivity, 0.01 to 0.2 absorbance unit full scale (AUFS); visible light source; ambient temperature; injection volume, 5–20 μL; wavelengths, 433, 490, and 505 nm.

(b) *Liquid chromatography column.*—E. Merck (Darmstadt, GFR) Hibar II LiChrosorb RP-18, 10 μm (4.6 mm × 25 cm), at ambient temperature.

(c) *Recorder.*—Varian (Palo Alto, CA) Aerograph Model 20. Parameters: single pen; 1 mV input; chart speed 0.25 in./min.

(d) *Filter system.*—Millipore (Bedford, MA) 0.45 μm vacuum filter apparatus.

(e) *Methanol.*—HPLC grade (J.T. Baker Chemical Co., Phillipsburg, NJ).

(f) *Water.*—Distilled, filtered through Millipore filter apparatus before use.

(g) *Tetra-n-butylammonium hydroxide (TBAH) solution.*—AR grade (Baker Chemicals Ltd, Poole, UK); 40% solution in water (1.54M). Store at ambient temperature in dark.

(h) *Potassium phosphate, monobasic.*—Baker analyzed reagent grade (J.T. Baker Chemical Co.).

(i) *Ion-pair reagent.*—To 52 mL 1.11M KH_2PO_4, add 25 mL 1.54M TBAH solution, to make 77 mL 0.5M tetra-*n*-butylammonium phosphate (TBAP), pH 7.2. Filter TBAP reagent through Millipore apparatus and store in capped brown bottle in dark when not in use.

(j) *Eluants.*—Mobile phases (degassed) consist of methanol–water at ratios (v/v) of (60 + 40), (56.5 + 43.5), or (45 + 55), each containing 0.005M TBAP. Filter all eluants and samples through Millipore filter before use. Before sample analyses are performed, equilibrate

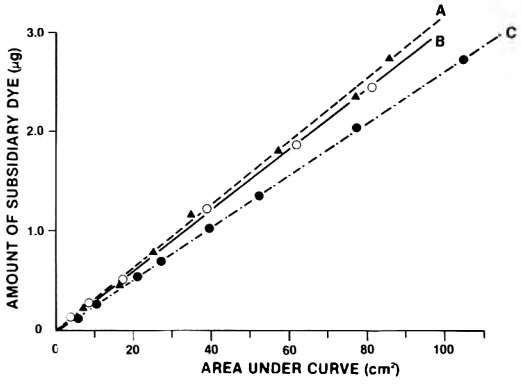


Figure 4. Peak area as a function of quantity of subsidiary standard injected. Conditions as described in text. Subsidiary A, Fast Red E; subsidiary B, 1-*p*-sulfophenylazo-2-naphthol-3,6-disulfonic acid, trisodium salt; subsidiary C, 3-carboxy-5-hydroxy-1-*p*-sulfophenyl-4-phenylazo-pyrazole, disodium salt.

chromatographic system ca 20–30 min each day. No special conditioning procedures are required for new columns. At end of each day, thoroughly rinse chromatographic system with 100 mL water followed by ca 25 mL methanol. This rinsing is an important protection for chromatographic system.

(k) *Reference compounds.*—Commercial samples of Amaranth, Sunset Yellow FCF, and Tartrazine, as well as samples of Fast Red E and the major subsidiary dye of Sunset Yellow FCF were available from the Food Colour Unit of the Food Research Division. The primary dyes were used as supplied and the 2 subsidiaries were purified by recrystallizing twice from ethanol–water. Purity of the Fast Red E was spectrophotometrically determined to be 98.9% calculated with an absorptivity of 41.3 at 505 nm (23). Similarly, with an absorptivity of 50.1 at 490 nm (23), the purity of the Sunset Yellow FCF subsidiary was calculated to be 91.0%. It was determined that excess salt from the synthesis was responsible for low dye content before recrystallization.

The required subsidiary of Tartrazine was not commercially available and thus synthesis was necessary. Redistilled aniline was acidified with HCl, cooled, and then diazotized by using sodium nitrite solution. The diazonium solution, after the addition of sodium hydroxide, was chilled and coupled with recrystallized 1-(4-sulfophenyl)-3-carboxy-5-pyrazolone. The subsidiary was then filtered, dried, and stored. Because the subsidiary remained slightly acidic, solutions prepared for HPLC were first neutralized by dropwise addition of 1% NaOH before ion-pair reagent was added. Identity of the subsidiary was confirmed by TLC, spectrophotometry, and HPLC. Purity, calculated with an absorptivity of 57.6 at 433 nm (23), was 89.7%.

Analysis of Commercial Samples

Accurately weigh ca 0.25 g Amaranth or Sunset Yellow FCF, or 1.5 g Tartrazine (subsidiary dye content of commercial Tartrazine is generally much lower than in Amaranth and Sunset Yellow FCF), and quantitatively transfer to 100 mL volumetric flask. Dilute to volume with distilled, filtered water to completely dissolve dye. Pipet 1.0 mL 0.5M TBAP ion-pair reagent plus 10.0 mL of dye solution into second 100 mL volumetric flask and dilute to volume with distilled water. Mix thoroughly and use this solution for HPLC analysis.

Select appropriate wavelength (see *Apparatus and Reagents*, (k)) then inject 20 μL sample and, using suitable detector sensitivities (between 0.01 and 0.2 AUFS depending on quantity injected), obtain chromatograms of primary and subsidiary dyes.

Calibration with Standards

Accurately weigh ca 0.13 g subsidiary dye and transfer to 100 mL volumetric flask. Dilute to volume with distilled, filtered water and completely dissolve dye.

Into separate 100 mL volumetric flasks, transfer (a) 10.0 mL and (b) 2.0 mL subsidiary dye solution. Add 1.0 mL 0.5M TBAP ion-pair reagent to each flask and dilute to volume with distilled, filtered water. Mix thoroughly.

Select wavelength required and equilibrate column with appropriate eluant. Inject, separately, 5, 10, 15, and 20 μL of each solution, obtain chromatograms, and construct calibration curve of peak area (peak height times width at one-half

Table 1. Recovery of subsidiary dye (1-(4-sulfo-1-naphthylazo)-2-naphthol-6-sulfonic acid, disodium salt) added to Amaranth [a]

Added, ng	Added, %	Recd [b] ng	Recd, [b] %
10.0	1	9.66	96.6
15.0	1.5	14.6	97.6
30.0	3	30.6	102
40.0	4	40.0	99.9
50.0	5	47.1	94.2
160	16	147	92.1

[a] Based on 1 μg Amaranth injected.
[b] Averages of duplicate or triplicate injections.

Table 2. Recovery of subsidiary dye (1-*p*-sulfophenylazo-2-naphthol-3,6-disulfonic acid, trisodium salt) added to Sunset Yellow FCF [a]

Added, ng	Added, %	Recd,[b] ng	Recd,[b] %
20.0	1	20.4	102
30.0	1.5	30.0	100
60.0	3	60.6	101
80.0	4	100	100
100	5	100	100
140	7	141	101

[a] Based on 2 μg Sunset Yellow FCF injected.
[b] Averages of duplicate or triplicate injections.

peak height) vs quantity of subsidiary (Figure 4). Eight or more calibration points are obtained for each dye. A second calibration curve is obtained when samples of primaries spiked with subsidiaries are analyzed for percentage recovery.

Recovery Studies

Prepare solutions for recovery studies by using spiked samples of commercial dyes. Dyes selected had little or undetectable levels of subsidiary dyes present and were analyzed to determine background. Amount of background subsidiary dye may be calculated by using the calibration curve obtained from standards. Analyze each subsidiary in duplicate, at a minimum of 6 levels and at least once at specification limit level of 4.0% for Amaranth, 5.0% for Sunset Yellow FCF, and 1.0% for Tartrazine. Prepare various stock solutions of the dyes and add appropriate quantities to sample solution. All solutions should contain 0.005M ion-pair reagent. Plot calibration curve of peak area (peak height times width at one-half peak height) as a function of quantity of subsidiary dye and use this to calculate quantity of subsidiary in the commercial dye samples.

Table 3. Recovery of subsidiary dye (3-carboxy-5-hydroxy-1-*p*-sulfophenyl-4-phenylazo-pyrazole, disodium salt) added to Tartrazine [a]

Added, ng	Added, %	Recd,[b] ng	Recd,[b] %
18.0	0.06	16.8	93.1
21.0	0.07	20.3	96.6
27.0	0.09	26.7	98.8
39.0	0.13	38.8	99.7
150	0.5	150	100
300	1.0	298	99.3

[a] Based on 30 μg Tartrazine injected.
[b] Averages of duplicate or triplicate injections.

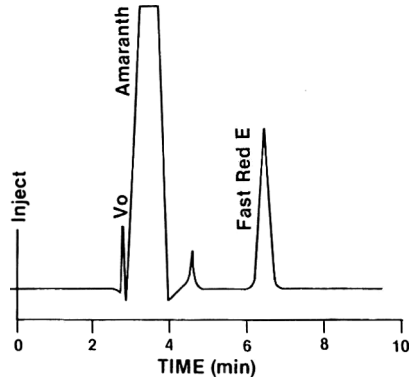


Figure 5. Chromatogram of commercial Amaranth spiked at 1.0% with Fast Red E. Conditions as described in text. Mobile phase methanol-water (60 + 40) containing 0.005M TBAP. Wavelength 505 nm.

Results

Calibration Data

The quantity of subsidiary dye injected as a function of peak area is illustrated in Figure 4. The quantity of standard injected was 7.4 ng–2.4 μg of Fast Red E, 20 ng–2.5 μg of the Sunset Yellow FCF subsidiary, and 17 ng–2.7 μg of the Tartrazine subsidiary. In all cases, the subsidiaries were well resolved and the plots were linear.

Recovery Data

The recovery data are presented in Tables 1–3. Quantities of Amaranth injected ranged from

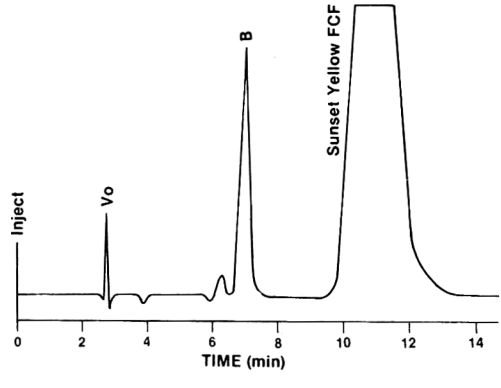


Figure 6. Chromatogram of commercial Sunset Yellow FCF spiked at 1.0% with (B) 1-*p*-sulfophenylazo-2-naphthol-3,6-disulfonic acid, trisodium salt. Conditions as described in text. Mobile phase methanol-water (45 + 55) containing 0.005M TBAP. Wavelength 490 nm.

Table 4. Subsidiary dye [a] content of some commercial dyes

Sample		Reported by manufacturer, [b] %	Detd by this method, %
Amaranth	A	0.2	0.1
	B	0.8	0.5
	C	1.0	0.99
	D	1.4	1.5
Sunset Yellow FCF	A	4.9	4.7
	B	3.8	3.7
	C	4.7	4.8
	D	ND [c]	ND
	E	3.0	2.6
Tartrazine	A	0.2	0.12
	B	<0.3	0.053
	C	<1.0	0.006

[a] 1-(4-Sulfo-1-naphthylazo)-2-naphthol-6-sulfonic acid, disodium salt in Amaranth; 1-*p*-sulfophenylazo-2-naphthol-3,6-disulfonic acid, trisodium salt in Sunset Yellow FCF; 3-carboxy-5-hydroxy-1-*p*-sulfophenyl-4-phenylazo-pyrazole, disodium salt, in Tartrazine.

[b] Commercial analyses by TLC except for Tartrazine A which was analyzed by HPLC.

[c] ND = none detected.

0.75 to 4.1 μg, Sunset Yellow FCF from 1.2 to 4.6 μg, and Tartrazine from 4.6 to 31 μg. Chromatograms shown in Figures 5, 6, and 7 are representative of results obtained at the lowest spiking levels studied, as indicated in Tables 1–3.

Discussion

Recoveries ranged from 92 to 102% for 1-(4-sulfo-1-naphthylazo)-2-naphthol-6-sulfonic acid, disodium salt in Amaranth; 100–102% for 1-*p*-sulfophenylazo-2-naphthol-3,6-disulfonic acid, trisodium salt in Sunset Yellow FCF; and 93–100% for 3-carboxy-5-hydroxy-1-*p*-sulfophenyl-4-phenylazopyrazole, disodium salt in Tartrazine. Corrections were made for any subsidiary dye originally contained in the primary standards.

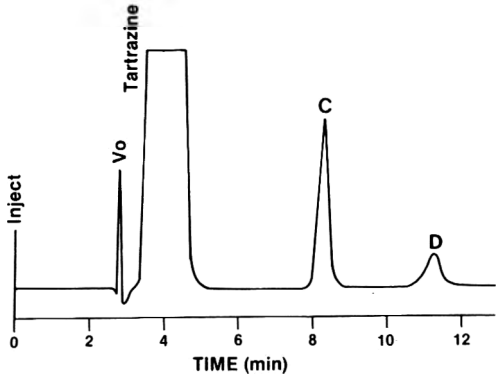


Figure 7. Chromatogram of commercial Tartrazine spiked at 0.06% with (C) 3-carboxy-5-hydroxy-1-*p*-sulfophenyl-4-phenylazopyrazole, disodium salt. Conditions as described in text. Mobile phase methanol–water (56.5 + 43.5) containing 0.005M TBAP. Wavelength 433 nm. Peak (D) is probably another subsidiary, 3-carboxy-5-hydroxy-1-phenyl-4-*p*-sulfophenylazopyrazole.

Detection limits were not accurately determined for these subsidiaries because there was no need to develop methodology for less than one-tenth the permitted maximum level. However, we estimate that the method as developed is quite capable of detecting at least 1–2% of the permitted maximum.

Table 4 compares results obtained for a number of samples by the ion-pair HPLC method with those obtained by the manufacturer by using TLC and spectrophotometry or HPLC with ion exchange. It can be seen that the methods are generally in good agreement.

Although a different mobile phase was used for each subsidiary, Tartrazine and its subsidiaries were also resolved with methanol–water (60 + 40). Therefore, all 3 subsidiaries studied could be resolved with only 2 different eluants. However, the (56.5 + 43.5) combination maximized separation and resolution of Tartrazine and its subsidiaries.

During the development of this method, we observed that other peaks, presumably other subsidiaries of each primary dye, were resolved, indicating the possible application of the method to the determination of a number of subsidiary dyes for a more "total" picture. This ion-pair HPLC technique is now being evaluated for the determination of color intermediates.

References

(1) The Food and Drug Act and Regulations, Division 6, Department of National Health and Welfare,

Ottawa, Ontario, Canada
(2) Dolinsky, M. (1951) *J. Assoc. Off. Agric. Chem.* **34,** 411–416
(3) Stein, C. (1969) *J. Assoc. Off. Anal. Chem.* **52,** 34–38
(4) Stein, C. (1970) *J. Assoc. Off. Anal. Chem.* **53,** 677–681
(5) Bell, S. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 717–718
(6) Van Peteghem, C., & Bijl, J. (1981) *J. Chromatogr.* **210,** 113–120
(7) Link, W. B. (1961) *J. Assoc. Off. Agric. Chem.* **44,** 43–53
(8) Singh, M. (1970) *J. Assoc. Off. Anal. Chem.* **53,** 250–251
(9) Bell, S. (1973) *J. Assoc. Off. Anal. Chem.* **56,** 947–949
(10) Singh, M. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1067–1069
(11) Official Method FO 10, October, 1981, Health Protection Branch, Ottawa, Ontario, Canada
(12) *Official Methods of Analysis,* (1970) 11th Ed., AOAC, Arlington, VA, secs **34.085–34.086**
(13) Singh, M. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 48–49
(14) Bailey, J. E. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 565–571
(15) Wittmer, D. P., Nuessle, N. O. & Haney, W. G., Jr, (1975) *Anal. Chem.* **47,** 1422–1423
(16) Lawrence, J. F., Lancaster, F. E. & Conacher, H. B. S. (1981) *J. Chromatogr.* **210,** 168–173
(17) Puttemans, M. L., Dryon, L., & Massart, D. L. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1–7
(18) Aitzemüller, K., & Arzberger, E. (1979) *Z. Lebensm. Unters. Forsch.* **169,** 335–338
(19) Chudy, J., Crosby, N. T., & Patel, I. (1978) *J. Chromatogr.* **154,** 306–312
(20) Boley, N. P., et al. (1980) *Analyst* **105,** 589–599
(21) Calvey, R. J., Goldberg, A. L., & Madigan, E. A. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 665–669
(22) Bailey, C. J., Cox, E. A., & Springer, J. A. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1404–1414
(23) Venkataraman, K. (1977) *The Analytical Chemistry of Synthetic Dyes,* Wiley Interscience, New York, NY, pp. 484–485

DRUG RESIDUES IN ANIMAL TISSUES

High Pressure Liquid Chromatographic Determination of Sulfamethazine in Pork Tissue

BYRON L. COX and LEO F. KRZEMINSKI
The Upjohn Co., Agricultural Division, Kalamazoo, MI 49001

A high pressure liquid chromatographic (HPLC) method has been developed for determination of sulfamethazine residues in pork liver, kidney, muscle, and fat. The sample was extracted with acetone-chloroform, concentrated in the presence of dilute HCl, and partitioned between dilute HCl and hexane. The acid solution was washed with methylene chloride and then buffered with trisodium citrate and sodium hydroxide to pH 5.8-5.9. Sulfamethazine was extracted from the aqueous mixture with methylene chloride, concentrated, dissolved in buffer, and eluted from XAD-2 resin with methanol. Sulfamethazine was reliably quantitated at 0.1 ppm by HPLC on a Zorbax ODS column with detection at 254 nm with no interference from tissues or reagents. The average recovery from the edible tissues, i.e., liver, muscle, kidney, and fat, fortified at 0.1-0.4 ppm was 85.6 ± 3.7%.

Sulfamethazine, 4-amino-*N*-(4,6-dimethyl-2-pyrimidinyl) benzene sulfonamide, is used in the rearing of swine to prevent and treat disease as well as to promote growth. Therefore, a sensitive and reliable method was needed to monitor for drug residues. Early analytical methods were based on the Bratton-Marshall reaction (1) which was later modified for use in milk and tissues (2). Later, several thin layer chromatographic systems (3, 4) were reported in the literature, as well as the more selective gas-liquid chromatographic (5) and liquid chromatographic (6, 7) systems. The more recent publication describes a qualitative and quantitative gas chromatographic-mass spectrometric procedure specifying a stable isotope as the internal standard (8).

Because the previous described methods (5, 6, 8, 9) used derivatization steps which are time consuming and difficult to perform with biological extracts, a method that could quantitate sulfamethazine directly was desirable. Therefore, this paper describes a direct high pressure liquid chromatographic (HPLC) method that is sensitive, reliable, and capable of measuring sulfamethazine in pork tissues at the established tolerance of 0.1 ppm (10) and with higher recoveries than the previously reported HPLC methods (6, 7).

Received November 25, 1981. Accepted March 23, 1982.

METHOD

Reagents

(a) *Extraction solvent.*—Combine equal volumes of chloroform and acetone for extraction of tissues.

(b) *Buffer.*—Weigh 3.40 g KH_2PO_4 and 3.55 g Na_2HPO_4 in 1 L deionized water (pH 6.8 phosphate buffer); standard buffer solutions pH 4.0 and 7.0.

(c) *HCl solution.*—Prepare 1N HCl by adding 83.5 mL 37% HCl to 1 L deionized water.

(d) *Sodium hydroxide* (2N).—Add 80 g pellets to 1 L deionized water.

(e) *Citrate solution.*—Prepare a saturated trisodium citrate solution in deionized water (solubility, 72 g/100 mL cold water).

(f) *Mobile phase solvent.*—Add 250 mL UV grade acetonitrile to 1 L graduated cylinder, and dilute to mark with deionized water.

(g) *XAD-2 resin.*—Pack 5 kg resin (Rohm & Haas or equivalent) into 110 mm × 120 cm column and wet with deionized water; successively elute with 3.0 gal. acetone, 2.0 gal. methanol, and 25.0 gal. deionized water. Regulate flow of all liquids at 60-65 mL/min throughout cleanup operation. Store wet material in large plastic bag or in containers with deionized water. Remove fines by successive decantation before sample use to maintain uniform column flow.

(h) *Stock standard solutions.*—Dry USP Reference Standard Sulfamethazine 2 h in oven at 105°C before weighing. Dissolve 100 mg in 100 mL acetone (1000 ppm, solution A). Dilute 10 mL solution A to 100 mL with UV acetonitrile (100 ppm, solution B). Dilute 10 mL solution B to 100 mL with UV acetonitrile (10 ppm, solution C). Dilute 10 mL solution C to 100 mL with UV acetonitrile (1.0 ppm, solution D).

(i) *Liquid chromatographic standards.*—Rotoevaporate 1.0, 2.0, and 4.0 mL aliquots of solution D (1.0 ppm) in separate 100 mL round-bottom flasks. Add 8.0 mL mobile phase to each flask.

0004-5756/82/6506-1311-05$01.00

Standards are equivalent to 0.1, 0.2, and 0.4 ppm in 10 g tissue.

Apparatus

(a) *Adaptors.*—24/40, No. 5225-10, and 24/40, No. 5204 (Ace Glass, Inc., or equivalent).

(b) *Blender.*—Waring blender, 1 L glass bowl, refitted with gaskets cut from polyethylene freezer containers.

(c) *Liquid chromatograph.*—Pump System, DuPont Model 810; precision LDC Photometer UVIII Monitor (Model 1203), operated at 0.064 AUFS at 254 nm; Zorbax ODS, 4.6 mm × 25 cm column (No. 850952702, DuPont); and Valco liquid loop injector (No. CV-6-HPax, 6-port, 3000 psi) equipped with 20 μL loop; 1 mV Hewlett-Packard recorder (No. 7131A). Use of 1 mV recorder for 10 mV output from detector gives effective attenuation of 0.0064 AUFS. A 20 μL injection of 0.125 ppm standard sulfamethazine (2.5 ng on column) should give a 20 mm or greater peak height response and a retention time of 7–8 min.

(d) *Reservoir.*—250 mL round-bottom flask constructed with 24/40 male joint at bottom.

(e) *Chromatographic column.*—Glass, 15 × 300 mm, equipped with sintered disk and Teflon stopcocks.

(f) *Funnels.*—150 mL medium porosity fritted glass funnels.

(g) *Vacuum flask.*—500 mL side-arm vacuum flask.

(h) *Rotary evaporator.*—Rinco rotary evaporator and water bath set at 40–45°C.

(i) *pH Meter.*—Beckman Zeromatic Model SS-3, standardized with pH 7.0 buffer solution.

(j) *Separatory funnel.*—125 mL pear-shape, equipped with Teflon stopcocks.

(k) *Column guard.*—RP-18 MPLC™ Guard Column, Brownlee Labs.

Sample Preparation

Place 10 g thawed tissue in Waring blender jar, add 150 mL extraction solvent, and blend 5 min at low speed. Place 3–4 g Celite into 150 mL medium porosity funnel and wash with 25 mL acetone. Discard wash. Filter homogenate through Celite pad with vacuum into 500 mL filter flask. Rinse blender bowl with acetone to remove residue and add rinse to funnel. Transfer filtrate to 500 mL round-bottom flask, add 10 mL 1N HCl solution, and evaporate to 5–7 mL (11). (Note: Solvent removal is complete when bubbling stops; longer periods of evaporation will cause losses.)

Add 75 mL hexane via special adaptor (No. 5225) to 500 mL flask, swirl, and transfer hexane–HCl solution to 125 mL separatory funnel. Stopper and invert gently 10–12 times and let phases separate. (Note: For fat samples, after evaporation and addition of hexane, warm flask in hot tap water to dissolve residue before transfer to separatory funnels.)

Drain lower HCl solution into clean 125 mL separatory funnel and hold. Add 5 mL 1N HCl solution to 500 mL sample flask, swirl, and transfer to hexane in separatory funnel, stopper, and invert 30 times. Let phases separate. Drain lower layer into second funnel containing HCl solution. Repeat this operation a second time with 5 mL 1N HCl. Discard funnel containing hexane.

Add 15 mL methylene chloride to 500 mL sample flask, swirl, and transfer to separatory funnel containing combined HCl solutions. Stopper and shake vigorously 30 s; vent at least once during this period. Let phases separate and discard lower methylene choride layer.

Drain HCl solution from funnel into clean 250 mL round-bottom flask, wash separatory funnel with 3–5 mL deionized water, and add rinse to sample flask. Add 25 mL saturated trisodium citrate to 500 mL sample flask, swirl, and transfer to 125 mL separatory funnel, then stopper, shake 4–5 times, and drain into HCl solution in 250 mL flask. Rinse walls of separatory funnel thoroughly with deionized water and add this rinse to the 250 mL flask.

Adjust HCl–trisodium citrate solution on pH meter to pH 5.8–5.9 with 2N NaOH.

Transfer pH-adjusted solution back to separatory funnel and extract with four 15 mL portions of methylene chloride. Shake each 15 mL portion vigorously for 30 s and let phases separate. (Note: Check pH of buffered aqueous phase after first extraction and, if necessary, re-adjust to pH 5.8 with 2N NaOH.) Evaporate the 4 combined methylene chloride fractions to dryness.

Dissolve residues from liver, kidney, and muscle in 10 mL of pH 6.8 phosphate buffer, swirl to ensure contact with glass surface, and proceed to XAD-2 column chromatography step. In the case of fat, omit column chromatography step. Dissolve residue in mobile phase and proceed to HPLC analysis.

Column Chromatography

Prepare 15 × 130 mm XAD-2 column, top with glass wool, and wash with 250 mL deionized water. Apply sample residue in 10 mL of pH 6.8 buffer. Rinse sample flask 3 times with 15 mL

portions of deionized water and transfer rinses to column. Repeat rinse and transfer with 100 mL deionized water. Discard all water washes. Add 100 mL methanol to sample flask, transfer to column, and elute sulfamethazine into clean 250 mL round-bottom flask. When flow of eluant stops, apply 3 psi nitrogen pressure to remove residual liquid. Add 10 mL 1N HCl to methanol eluate and carefully rotary-evaporate to dryness (caution: may bump).

Purge flask with nitrogen to remove any residual HCl fumes. Dissolve dried sample residue in 8.0 mL mobile phase.

Liquid Chromatography

Degas acetonitrile–deionized water (1 + 3) under vacuum. Operate chromatograph at ambient temperature with pressure at 1100 psi and attenuation at 0.0064 AUFS. Install Zorbax ODS 4.6 mm × 25 cm C-18 column with RP-18 column guard. Pipet 1.0 mL solution D (1.0 ppm) into 100 mL round-bottom flask, evaporate to dryness, and redissolve in 8.0 mL mobile phase (0.125 ppm). Inject 20 μL aliquots of sample and standard with loop injector. Measure standards and samples to nearest 0.5 mm. (Retention time of sulfamethazine under these conditions was approximately 7–8 min.)

Calculations

Calculate amount of sulfamethazine in the samples by the following equation:

$$\text{Sulfamethazine, ppm} = [(PH/PH') \times C \times (D/E)]/[\text{g sample} \times 1000]$$

where PH = peak height of unknown, mm; PH' = peak height of standard, mm; C = ng standard injected on column; D = final volume of sample, mL; E = aliquot injected, mL; 1000 = factor to convert ng to μg.

Fortification of Tissues

To 10 g control liver, kidney, muscle, or fat, pipet 1.0, 2.0, or 4.0 mL standard (D, 1.0 ppm) into Waring blender and immediately proceed with extraction of sample with 150 mL solvent as described.

Results and Discussion

The overall recoveries of sulfamethazine from tissues fortified in triplicate at 0.1, 0.2, and 0.4 ppm were 82.9 ± 2.2% for liver, 85.0 ± 2.5% for kidney, 83.9 ± 2.7% for muscle, and 90.6 ± 1.6% for fat (Table 1). The average recovery over the 4 tissues was 85.6 ± 3.7%. No interfering peaks were observed at the retention time of sulfamethazine in any of the control tissues (Figure 1). Peak height responses of the standards were linear from 0.1 to 0.4 ppm.

During the early development of the method, low recoveries of sulfamethazine (35 to 40%)

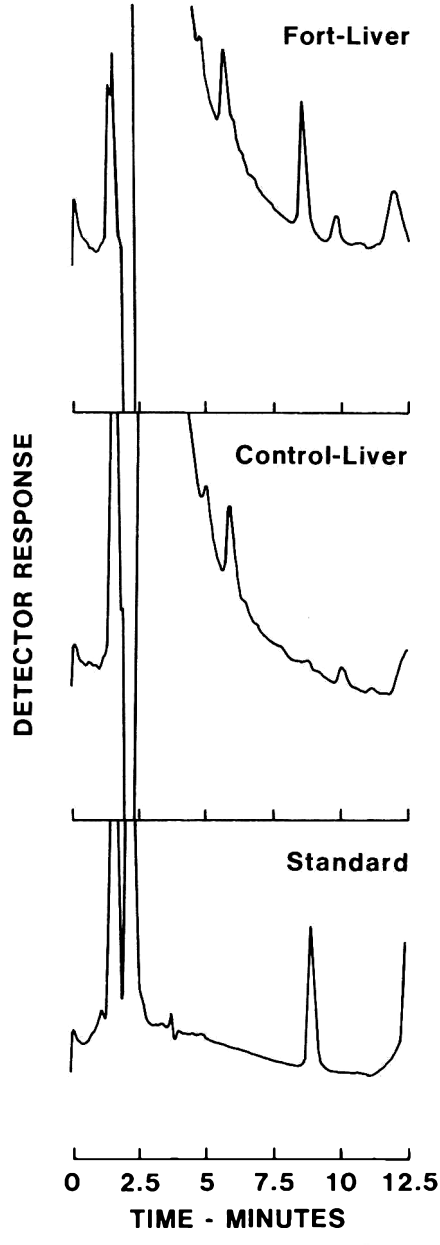


Figure 1. Typical chromatogram: sample in 8.0 mL mobile phase; standard concentration 0.125 ppm in mobile phase; injection 20 μL or 2.5 ng on column or equivalent to 0.1 ppm in tissue. Fortified tissue was equivalent to 0.1 ppm sulfamethazine.

Table 1. Percent recovery of sulfamethazine from fortified control pork tissues: liver, kidney, muscle, and fat

Tissue	Fortification level, ppm 0	0.1	0.2	0.4	Overall[a]
Liver	NP[b]	83.3	85.4	83.3	
	NP	84.6	78.8	84.4	
	NP	83.3	79.6	83.1	82.9 ± 2.2
Kidney	NP	85.2	88.8	87.6	
	NP	80.8	84.6	85.4	
	NP	84.9	85.7	81.9	85.0 ± 2.5
Muscle	NP	82.1	84.7	86.4	
	NP	80.8	83.7	84.6	
	NP	85.2	88.0	79.4	83.9 ± 2.7
Fat	NP	92.6	88.9	90.7	
	NP	89.3	88.4	90.1	
	NP	91.1	90.9	93.1	90.6 ± 1.6

[a] Average over all tissues and all levels was 85.6 ± 3.7%.
[b] No peak at retention time of sulfamethazine.

were obtained when fortification of the liver, kidney, muscle samples were done in the Waring blender at the start of the procedure.

In an attempt to discover where losses occurred, a sample extract was fortified after the final cleanup step and before the HPLC analysis. Recovery was approximately 50%. This demonstrated that the final residue contained a substance that in some way interacted with the sulfamethazine and caused low values. Addition of the XAD-2 resin chromatography step removed the interfering substance and increased the recoveries of samples fortified in the Waring blender from 35 to 85%. This column chromatography step was not necessary to obtain good recoveries of sulfamethazine from fat tissue.

To evaluate the procedure with actual in vivo residues, analysis of liver, kidney, muscle, and fat from swine fed a diet containing 44 g lincomycin and 110 g sulfamethazine per metric ton for 28 days showed that the tissues contained residues >0.1 ppm (0 day withdrawal) but residues decreased rapidly (Table 2). On treatment residues in liver were the highest at 5.8 ppm decreasing to 0.16 ppm 5 days post-treatment. Residues declined to <0.1 ppm in kidney, muscle, and fat at 5 days and in liver at 10 days post-treatment.

To ensure that the method was working properly throughout the study, a control and fortified control tissues were assayed with each day's run. The average overall recovery for these fortified tissues was within 2% of the previously reported data (Table 1).

Because all tissues were ground through a 2 mm plate and then stored in the freezer (−20°C) until they were assayed, a stability study was carried out. Although only a limited number of samples were run, the results showed that after 15 days of freezer storage, sulfamethazine had degraded by 13.9% in the muscle and 12.6% in the liver (Table 3). These results are 4–6% higher, respectively, than those reported in previously published data (12). Sulfamethazine stability in

Table 2. Observed ppm sulfamethazine residue in pork tissue during withdrawal phase[a]

Tissue	Time after last feeding 0 Days[b]	5 Days[c]	10 Days	15 Days
Liver	5.8 ± 2.9	0.16 ± 0.04	<0.1	<0.1
Kidney	2.2 ± 0.4	<0.1	<0.1	—
Muscle	1.2 ± 0.4	<0.1	<0.1	—
Fat	1.3 ± 0.2	<0.1	<0.1	—

[a] Average of 4 animal values, uncorrected for storage loss and recovery; see Table 3.
[b] Individual animal values: liver, 4.6, 8.7, 7.5, 2.2
kidney, 2.6, 1.9, 1.8, 2.5
muscle, 1.6, 0.8, 1.3, 1.0
fat, 1.2, 1.6, 1.1, 1.2
[c] Individual animal values: liver, 0.1, 0.17, 0.2, 0.16.

Table 3. Stability of sulfamethazine in pork liver and muscle at −20°C [a]

Tissue	0 Days	7 Days	15 Days	Lost, % [b]
Liver	79.4	79.9	67.6	
Liver	76.2	79.2	68.3	
Av.	77.8	79.6	68.0	12.6
Muscle	79.4	78.4	66.2	
Muscle	79.4	76.8	70.5	
Av.	79.4	77.6	68.4	13.9

[a] Samples of control tissues (10 g) were fortified with 0.5 mL acetone solution containing 10 μg/mL (solution C) and stored in freezer until assays. Values reported are uncorrected for recovery.

[b] Average of 15-day stability values divided by 0-day stability was used to obtain percent sulfamethazine lost during storage interval.

kidney and fat was not studied because the published data indicated smaller losses of sulfamethazine in these frozen tissues. No correction for stability loss was applied to the values in the swine residue data (Table 2), because no tissues were frozen more than 10 days before assay, and if the correction values were applied, they would not exceed the 0.1 ppm tolerance.

Acknowledgments

The authors express their appreciation to R. E. Gosline and C. J. Subacz for the collection and preparation of tissue extracts.

References

(1) Bratton, A. C., Marshall, E. K., Jr, Babbitt, D., & Hendrickson, A. R. (1939) *J. Biol. Chem.* **128,** 537–550

(2) Tishler, F., Sutter, J. L., Bathish, J. N., & Hagman, H. E. (1968) *J. Agric. Food Chem.* **16,** 50–53

(3) Phillips, W. F., & Trafton, J. E. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 44–47

(4) Woolley, J. L., Jr, Murch, O., & Sigel, C. W. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 545–549

(5) Goodspeed, D. P., Simpson, R. M., Ashworth, R. B., Shafer, J. W., & Cook, H. R. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1050–1053

(6) Johnson, K. L., Jeter, D. T., & Claiborne, R. C. (1975) *J. Pharm. Sci.* **64,** 1657–1660

(7) Vilim, A. B., Larocque, L., & MacIntosh, A. I. (1980) *J. Liq. Chromatogr.* **3,** 1725–1736

(8) Suhre, F. B., Simpson, R. M., & Shafer, J. W. (1981) *J. Agric. Food Chem.* **29,** 727–729

(9) Manuel, A. J., & Steller, W. A. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 794–799

(10) *Code of Federal Regulations* (1979) Title 21, Food and Drug Part 556

(11) Gosline, R. E., Krzeminski, L. F., & Neff, A. W. (1969) *J. Agric. Food Chem.* **17,** 1148–1149

(12) Saschenbrecker, X., & Fish, A. (1980) *Can. J. Comp. Med.* **44,** 338–345

MEAT AND MEAT PRODUCTS

Screening Procedure for Detection of Volatile *N*-Nitrosamines in Cooked Bacon by One-Trap Mineral Oil Vacuum Distillation and Thermal Energy Analyzer

EARL L. GREENFIELD, G. A. VASCO, L. LEGETTE, and ANTHONY MALANOSKI

Food Safety and Inspection Service, Chemistry Division Laboratory Branch, Beltsville, MD 20705

A rapid screening procedure for the detection of volatile *N*-nitrosamines in fried bacon has been developed for the regulatory monitoring of bacon. This procedure uses vacuum mineral oil distillation of an alkaline sample. The distillate is collected in a prewet trap immersed in liquid nitrogen. After thawing, the distillate is transferred to a separator. The trap is rinsed with methylene chloride which is then used to extract the nitrosamines. The methylene chloride is dried with Na_2SO_4 and concentrated. A gas-liquid chromatograph coupled to a thermal energy analyzer is used to identify and quantitate the nitrosamines. Recoveries of 7 volatile *N*-nitrosamines added to 25 g fried bacon at the 10 ppb level (μg/kg) ranged from 78 to 92%.

The occurrence of volatile nitrosamines in cooked bacon has been well documentated by Fazio (1), Sen (2, 3), Fiddler (4), Crosby (5), Gough (6), and many others. Because nitrosamine compounds are known carcinogens, the Food Safety and Inspection Service established an action level (7) of 10 ppb. The action level made it imperative that a rapid screening method for nitrosamines be developed, because the multidetection method developed by the Food and Drug Administration (8) was not adequately productive for a monitoring program. Of the many analytical methods developed for the detection of volatile nitrosamines, the mineral oil distillation method in conjunction with the thermal energy analyzer (TEA) reported by Fine et al. (9) seemed to be the most feasible. A comparison of the Fine and FDA methods conducted by Havery et al. (10) had demonstrated equivalency.

A preliminary study of the method was conducted with 3 industry laboratories. The basic method had not been subjected to either optimization or ruggedness testing; therefore, a prestudy conference was held to prepare a consensus method for study. A major addition to the method was the use of dipropylnitrosamine as an internal standard to be evaluated as a quality control tool to track the completeness of the vacuum distillation.

Each laboratory agreed to perform a total of 35 analyses: 30 samples of cooked, nitrite-free bacon fortified at a nominal 10 ppb level for each of 6 nitrosamines, dimethylnitrosamine (DMNA) diethylnitrosamine (DENA) dibutylnitrosamine (DBNA), nitrosopiperidine (NPIP), nitrosopyrrolidine (NPYR), and nitrosomorpholine (NMOR), and 5 samples consisting of 2 blanks and 3 samples spiked at 3, 8, and 13 ppb, respectively. Each laboratory added the internal standard to each sample. All nitrosamine standards used in the study were obtained from J. Keith of the Illinois Institute of Technology Research Institute.

Data from the first 25 analyses were evaluated to determine the effectiveness of the internal standard as a quality control tool. The consensus of the participating chemists was that it was effective and that all analyses with recoveries less than 70% or more than 110% should not be included in the statistical evaluation. Statistical analysis by the technique of Steiner (11) was performed. To simplify the statistical analysis, only those samples where all 4 laboratories reported acceptable internal standard recoveries were pooled; Table 1 is a compilation of the repeatability statistical data with coefficients of variation from 9.5 to 20.0 for the different nitrosamines (12 samples).

The method was recommended for interim monitoring use until it could be optimized, tested for ruggedness, and subjected to a full collaborative study from which better performance standards could be developed.

The method was then subjected to the ruggedness test as described by Youden (11).

Received June 18, 1981. Resubmitted February 8, 1982. Accepted June 17, 1982.

Presented at the 94th Annual Meeting of the AOAC, Oct. 20–23, 1980, at Washington, DC.

0004-5756/82/6506-1316-03$01.00

Table 1. Compilation of statistical data for correlation study (ppb)

Statistic[a]	DMNA	DENA	DBNA	DPNA	NPIP	NPYR	NMOR
n	48	48	48	48	48	48	48
$\bar{x}$	7.79	9.18	5.56	8.30	5.01	13.54	14.58
s_0	1.07	0.87	1.11	0.86	0.66	2.05	2.16
CV_0	13.7	9.5	20.0	10.4	13.2	15.6	14.8
s_x	1.52	1.06	1.12	1.03	0.66	2.52	2.52
CV_x	19.51	11.5	20.1	12.4	13.2	18.6	17.3
s_0/s_x	0.704	0.821	0.991	0.835	1.00	0.813	0.857

[a] $\bar{x}$ = mean value reported, ppb; s_0 = repeatability standard deviation, ppb; s_x = reproducibility standard deviation, ppb.

Table 2. Optimization factors investigated

Parameter	Level	Level
Level of vac.	** 2 torr	5 torr
Time of vac. before heating	0 min	** 10 min
Number of traps used	** 1	2
* Adaptor side arm	insulation	** 170°–175°C heated
Number of systems on vac. single pump	1	** 2
* Max. temp. of distn	100°C	** 120°C
* Time on system vac. after heat removed from flask	0 min	** 15 min
* Time of ext concn from ca 85 mL to ± 4 mL, 60°C H_2O bath	0.5 h	** 1.0 h
* Final concn vol.	0.5 mL	** 1.0 mL
Concn using N-Evap	room temp.	** 40°C H_2O bath
* Temp. of trap on TEA	slurry −150°C	** Liq. N_2 −196°C

* Factors showing a significant influence.
** Factors incorporated in method revision.

Table 3. Statistical evaluation of one-trap method at 10 ppb

Statistic	DMNA	DENA	DPNA	DBNA	NPIP	NPYR	NMOR
$\bar{x}$, %	83.9	83.7	82.4	78.0	90.7	91.2	92.3
s_0	5.9	7.4	5.1	8.9	5.4	4.8	5.1
CV_0, %	8.8	8.8	6.2	11.4	5.9	5.3	5.6

[a] $\bar{x}$ = mean value reported (19 analyses); s_0 = standard deviation; CV = coefficient of variation.

The factors tested are listed in Table 2. Five of the factors tested caused significant variations: heating of the adaptor side arm; maximum temperature of distillation; length of time the system was maintained on vacuum after the heat source was removed; concentration time; and final concentration volume.

The revised method was subjected to evaluation by 2 analysts. Commercially prepared nitrite-free bacon was used. Eighteen samples of the cooked bacon were spiked with a solution containing DMNA, DENA, DBNA, NPIP, NPYR, and NMOR at 10 ppb. DPNA was also added as an internal standard. The results are listed in Table 3; coefficients of variation for repeatability ranged from 5 to 9% compared with 9.5 to 20% for the correlation study. The method was considered ready for collaborative study (12).

References

(1) Fazio, T., White, R. H., Dusold, L. R., & Howard, J. W. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 919–921
(2) Sen, N. P., Donaldson, B., Iyengar, J. R., & Panalaks, J. (1973) *Nature* **241**, 473
(3) Sen, N. P., Iyengar, J. R., Donaldson, B., & Panalaks, T. (1974) *J. Agric. Food Chem.* **22**, 540–541
(4) Fiddler,W., *et al.* (1974) *J. Food Sci.*
(5) Crosby, N. T., Foreman, J. K., Palframan, J. F., &

Sawyer, R. (1972) *Nature* **342,**
(6) Gough, T. A., Goodhead, K., & Walters, C. L. (1976) *J. Sci. Food Agric.* **27,** 121 et seq.
(7) *Fed. Regist.* (1978) **43,** 20 992–20 995
(8) Fazio, T., Howard, J. W., & White, R. (1971) *Proceedings of Heidelberg Meeting on Nitrosamines*, International Agency for Research on Cancer, Lyon, France, pp. 16–24
(9) Fine, D. H., Rounbehler, D. P., & Oettinger, P. E. (1975) *Anal. Chim. Acta* **78,** 383–389
(10) Havery, D. C., Fazio, T., & Howard, J. W. (1977) *Proceedings of the Working Conference on Environmental Aspects of Nitrosamines*, University of New Hampshire, IARC Publication No. 19, International Agency for Research on Cancer, Lyon, France, pp. 41–52
(11) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of AOAC*, AOAC, Arlington, VA
(12) Greenfield, E. L., Smith, W. J., & Malanoski, A. J. (1982) *J. Assoc. Off. Anal. Chem.* 65, 1319–1332

Mineral Oil Vacuum Distillation Method for Nitrosamines in Fried Bacon, with Thermal Energy Analyzer: Collaborative Study

EARL L. GREENFIELD, WERTICE J. SMITH, and ANTHONY J. MALANOSKI
Food Safety and Inspection Service, Chemistry Division Laboratory Branch, Beltsville, MD 20705

Collaborators: M. Benson; W. Fiddler; M. Grabber; H. Herring; K. Hill; L. Legette; K. A. Meyer; M. Robach; M. Wolfe

Nine laboratories participated in a collaborative study of a method for determining 6 nitrosamines, dimethylnitrosamine, diethylnitrosamine, dibutylnitrosamine, nitrosopiperidine, nitrosopyrrolidine, and nitrosomorpholine, in the 5-17 ppb range. The coefficients of variation for repeatability were 10.8, 8.5, 10.4, 8.5, 8.7, and 7.8% with corresponding coefficients of variation for reproducibility of 16.4, 12.0,13.6, 10.8, 11.2, and 10.3% and recoveries of 89.6, 91.6, 84.7, 90.0, 89.6, and 88.1%, respectively. The method was adopted official first action.

The mineral oil vacuum distillation-thermal energy analyzer method reported by Greenfield (1) was collaboratively studied for 6 nitrosamines: dimethylnitrosamine (DMNA), diethylnitrosamine (DENA), dibutylnitrosamine (DBNA), nitrosopiperidine (NPIP), nitrosopyrrolidine (NPYR), and nitrosomorpholine (NMOR). The study was planned to cover the range of 5–17 ppb by fortifying cooked bacon which was prescreened so that only low levels of DMNA and NPYR were present. All bacon was cooked in an electric skillet, at 340°F. The pan was allowed to equilibrate and the bacon was fried 3 min on each side and turned only once. The cooked bacon was blotted to remove excess fat, cooled, and then ground to obtain a uniform sample. The fortification levels were a nominal 0, 5, 7, 10, 13, and 17 ppb for each nitrosamine. In addition, 3 samples of bacon with NPYR in the range of 10–18 ppb were included. The samples also contained low levels of DMNA, DENA, and NPIP at levels for the most part below the level of reliable measurement.

Samples were analyzed as either blind duplicates, triplicates, or quadruplicates. The additional replicate analyses were added at the request of the Mathematics and Statistics Division, FSIS, to obtain a larger data base for review. For the purposes of the collaborative study evaluation, it was determined, before any samples were analyzed, that only the first 2 analyses at each level would be used for statistical method evaluation. All values were used in the determination of method performance standards.

Samples of cooked bacon were forwarded to collaborators only after they had analyzed practice samples successfully. A set of collaborative samples consisted of 23 coded samples. Each collaborator was requested to analyze samples so that the order of analysis was consistent, i.e., analysis of identical levels on the same day in all laboratories so that the study would be completed in the minimum time.

Received June 18, 1981. Resubmitted February 8, 1982. Accepted June 17, 1982.

This report of the Associate Referee, E. L. Greenfield, 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 C and adopted by the Association. See *J. Assoc. Off. Anal. Chem.* 65, 374 (1982).

Volatile *N*-Nitrosamines in Fried Bacon

Mineral Oil Vacuum Distillation-Thermal Energy Analyzer Method

Official First Action

Caution: Extreme care should be exercised in handling nitrosamines or solns of nitrosamines. These compds are reported to be potent carcinogens.

24.C01 ***Apparatus***

Thoroly clean all glassware and rinse with CH_2Cl_2 before use.

(a) *Boiling flask.*—500 mL with thermometer well and 24/40 ₮ neck.

(b) *Thermometer.*—75 mm immersion, −20 to 150°, 1° subdivisions (Fisher Scientific Co. No. 14-585-5C, or equiv.).

(c) *Vapor traps.*—A. H. Thomas No. 9466-275, or equiv., fitted with "O" ring socket joints size 18/9 (Kontes Glass Co. No. K-671500, or equiv.).

(d) *Adapter.*—Kontes Glass Co. No. K-183000 with ₮ 24/40 inner joint at bottom fitted with

0004-5756/82/6506-1319-14$01.00

18/9 "O" ring ⅋ ground ball joint (Kontes Glass Co. No. K-671300, or equiv.).

(e) *Vacuum pump.*—25 L/min free air displacement, Welch Duo Seal 1400 B (Fisher Scientific Co. No. 1-096), or equiv.

(f) *Vacuum tubing.*—$^5/_{16}$ in. bore (Fisher Scientific Co. No. 74-175D).

(g) *Vacuum controller.*—Cartesian-type (Cole Parmer Instrument Co. No. C-909-00), or equiv.

(h) *McLeod gage.*—Shielded (Cole Parmer Instrument Co. No. C-903-00), or equiv.

(i) *Heating mantle.*—500 mL (Kontes Glass Co. No. K-72600, or equiv.).

(j) *Heating tape.*—Double element, type 12, ½ in. wide × 2 ft long (Kontes Glass Co. No. K-729500, or equiv.).

(k) *Dewar flask.*—350 mm deep × 110 mm id (Kontes Glass Co. No. K-611785, or equiv.).

(l) *Laboratory jack.*—Lab Lift (Fisher Scientific Co. No. 14-673-10), or equiv.

(m) *Auto transformer.*—Power stat type 3PN117B (A. H. Thomas. No. 9461-F-15), or equiv.

(n) *Funnel.*—Buchner, 60 mL, coarse porosity fritted disk (Kontes Glass Co. No. K-955000, or equiv.).

(o) *Evaporative concentrator.*—Kuderna-Danish (K-D), 250 mL 24/40 ⅋ top joint, 19/22 ⅋ lower joint (Kontes Glass Co. No. K-850500, or equiv.).

(p) *Concentrator tube.*—Size 425, 19/22 ⅋ top joint, 4 mL (Kontes Glass Co. Cat. No. K-570050) with 19/22 ⅋ stopper (K-850500).

(q) *Distillation column.*—Snyder, with 24/40 ⅋ joints, 3 sections, size 121 (Kontes Glass Co. No. K-503000, or equiv.).

(r) *Concentration equipment.*—N-Evap with H_2O bath No. A-11151, Teflon-covered needles No. 10603, and thermometer No. 1111 (Organomation Associates), or equiv.

(s) *Gas chromatograph.*—Shimadzu GC 4C PR 6, with automatic cooling, temperature programmer, injection port temp. programmer, automatic door opener, glass-lined heated transfer line from gas chromatograph to TEA, dual column system with differential flow controllers, pressure gauges, and rotometers; recorder R 11M, single pen, 1 mV full scale, or equiv. (Shimadzu Scientific Instruments, Inc., Oakland Ridge Industrial Center, Columbia, MD 21045).

Glass column, 2.7 m × 3.0 mm id (5.0 mm od) packed with 100–120 mesh Analab AB (Analabs, Inc.) coated with 10% Carbowax 20M and 5% KOH. To prep. packing, dissolve 5.0 g Carbowax 20M and 2.5 g KOH in 100 mL MeOH contained in 500 mL r-b flask, ⅋ 24/40 neck. Slowly add 42.5 g Anakrom AB, 100–120 mesh, while gently swirling flask. Place flask on rotary evaporator ca 5 min. Apply vac. slowly at first and then increase to ca 26 in. Hg as foaming or bubbling subsides.

Allow support to rotate until all MeOH is drawn off as indicated by tumbling of support. Release vac. slowly and remove flask from system. Place flask contg support in 100° gravity convection oven to remove traces of MeOH. Transfer coated support to jar with screw-cap lid until used. Pack column by incremental addn under light vac. while gently tapping with rod. When filled, insert glass wool plugs in ends. Condition packed column, unconnected from heated transfer line, 24 h at 225° with 40 mL N carrier gas/min. Conditions: injector 185°, column 165° isothermal, interface line from gas chromatograph to TEA 230°, N carrier gas 40 mL/min.

Operating parameters: Recorder response should be >10% for 1.5 ng *N*-nitrosopyrrolidine. Resolution (R) between *N*-nitrosopiperidine and *N*-nitrosopyrrolidine should not be <0.8 when calcd as follows:

$$R = (T_2 - T_1)/\tfrac{1}{2}(W_1 + W_2)$$

where T_1 and T_2 = retention times (mm) of *N*-nitrosopiperidine and *N*-nitrosopyrrolidine; W_1 and W_2 = peak width at base (mm) of *N*-nitrosopiperidine and *N*-nitrosopyrrolidine.

(t) *Thermal energy analyzer (TEA).*—Model 502 (Thermo Electron Corp., 85 First Ave, Waltham, MA 02154). Operate according to manufacturer's instructions except use liq. N as trap coolant.

24.C02 *Reagents*

(a) *Paraffin oil.*—Heavy, Saybolt viscosity 335/350, laboratory grade (Fisher Scientific Co. No. 0-120, or equiv.).

(b) *Dichloromethane.*—Distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.). Conc. 100 mL of each lot to 1.0 mL and check for interfering peaks on GLC/TEA system.

(c) *Sodium sulfate.*—Anhyd. (Mallinckrodt Chemical Works, or equiv.).

(d) *Carborundum.*—No. 12 granules (A. H. Thomas Co. No. 1590-D30, or equiv.).

(e) *Mixed N-nitrosamine reference std.*—Stock std soln of 5 μg each/mL isooctane: *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodipropylamine (NDPA),

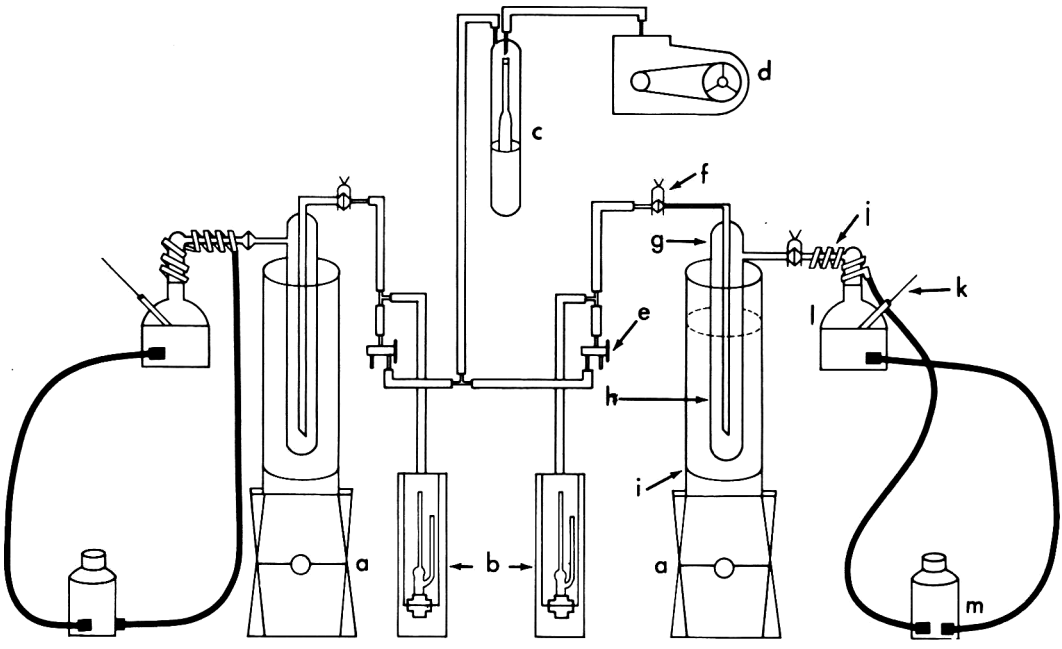


Figure 24:C1. Pumping and distillation assembly. (Dual system. Single distillation setup may be used.) a, laboratory jack; b, McLeod gage; c, vacuum controller; d, vacuum pump; e, 3-way stopcock; f, pinch clamp; g, vapor trap; h, liquid nitrogen; i, Dewar flask; j, heating tape; k, thermometer (−20° to 150 °); l, 500 mL boiling flask with thermometer well; m, power stat.

N-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR), and *N*-nitrosomorpholine (NMOR) (Chemical Repository, Illinois Institute of Technology Research Institute (IITRI), 10 W 35th St, Chicago, IL 60616).

(f) *Working mixed std soln.*—0.25 μg each nitrosamine/mL. Dil. stock soln 1:20 with CH_2Cl_2.

(g) *N-Nitrosodipropylamine (NDPA) internal std solns.—Stock soln:* 5 μg/mL isooctane (IITRI). *Working std soln:* 0.5 μg/mL. Dil. stock soln 1:10 with CH_2Cl_2. Use as internal std soln.

24.C03 *Sample Preparation*

Store fried bacon in −18° freezer overnite or in dry ice. Grind frozen sample thru 3/8 in. plate, mix thoroly, add sample thru grinder second time, and repeat mixing. Store sample in −18° freezer until analysis.

24.C04 *Distillation*

Assemble app. as in Figure **24:C1.**

Weigh 25.0 g sample in 500 mL r-b flask with thermometer well, add 2.0 mL 0.2N NaOH, 0.500 mL NDPA internal std soln, and 25.0 mL mineral oil. Place flask in heating mantle. Use adapter to connect boiling flask to prewet trap (2 mL H_2O) equilibrated in liq. N. Connect to vac. and check for leaks as system comes to operating pressure (<2 torr). Maintain vac. 10 min before applying heat. Wrap adapter with heating tape. Increase temp. in boiling flask, as indicated by thermometer in oil-filled well, from ambient to 120° in 55-60 min. Internal temp. of adapter is 170-175°. At end of heating period, lower heating mantle away from flask. Let flask cool while adapter remains heated 15 min (170-175°) with vac. maintained.

Carefully release vac. and lower Dewar flask away from trap. Disconnect flask. Invert adapter. Place trap with adapter attached in hood until contents are liq.

24.C05 *Transfer, Extraction, and Drying*

Rinse adapter with 10.0 mL CH_2Cl_2, collecting rinsing in trap. Remove adapter. Transfer distillate and CH_2Cl_2 washing to 125 mL separator. Wash trap by adding 15 mL CH_2Cl_2, 5 mL through stem and 10 mL to body. Rinse by shaking 1 min. Transfer CH_2Cl_2 rinsing to separator and ext by shaking 1 min. Let stand until phases sep.

Drain lower CH_2Cl_2 layer into second separator. Repeat trap washing and extn twice. Drain

pooled CH_2Cl_2 thru 30 g anhyd. Na_2SO_4 (held in 60 mL coarse fritted glass funnel prewetted with 25 mL CH_2Cl_2) into 250 mL K-D flask with 4 mL concentrator tube attached. Rinse second separator with 25 mL CH_2Cl_2 and drain rinse into K-D flask.

24.C06 ***Concentration***

Place one carborundum grain in concentrator tube, attach 3-ball Snyder column to K-D flask, and carefully conc. solv. to ca 4 mL on 60° H_2O bath in ca 1.5 h. Remove concentrator, wipe dry, and air-cool to ambient temp. Let remaining solv. in Snyder column drain into concentrator tube.

Transfer concentrator tube to N-Evap system. Reduce vol. to 1.0 mL in ca 30 min under gentle stream of N.

24.C07 ***Determination***

Inject 6.0 μL working mixed std soln contg 0.25 μg *N*-nitrosoamines/mL, into GLC app. coupled to TEA. Obtain retention time and response in ng/mm for each compound (see Figure **24:C2**).

Inject 6.0 μL concd sample ext. Obtain retention time and response of each compd present. (*Note:* Single std solns must be used to establish relative retention patterns.) If recovery of NDPA internal std soln is <70 or >110%, repeat analysis of sample.

Calc. amt of each nitrosamine in sample ext and det. amt in original sample, as follows:

For each nitrosamine, ng/g sample = (*PH*/*PH'*) × 10, where *PH* and *PH'* = peak ht in sample and std, resp.

Results and Discussion

The collaborative results for the individual nitrosamines are listed in Tables 1–6. Table 7 is the tabulation of the recoveries of the internal quality control standard, NDPA, at 10 ppb.

The averages, standard deviations, and the coefficients of variation (CV) were calculated for each level for each nitrosamine. For the levels of the study, the CV values of the raw data would normally be acceptable; however, because of the simplicity of the method we believed that one or more of the laboratories had not achieved proficiency. Even though all laboratories analyzed the practice samples successfully at residue levels, it may be necessary to increase the number of practice samples to obtain a true measure of proficiency.

Youden's ranking technique is not quite satisfactory for the evaluation of collaborative tests at residue levels. In addition, there is a need to identify standards of performance that any analyst should meet to determine that analytical proficiency has been achieved. These performance standards should be determined from the collaborative tests. Therefore, alternative methods were investigated for detecting performance outliers. Three techniques (2) were used: (*1*) the least squares linear regression, (*2*) a mean ratio, and (*3*) a coefficient of variation based on ratios. No single technique will establish performance standards or evaluate all parameters. The 3 techniques must be used as a battery of tests.

(*1*) The number of samples in the study, 2 blind replicates at 6 levels, suggested the use of the least squares linear regression as a means of evaluating acceptability of results. The regression equation and the correlation coefficient were generated for each nitrosamine and each collaborator. The correlation coefficients (Table 8) appeared to be consistent for DMNA, DENA,

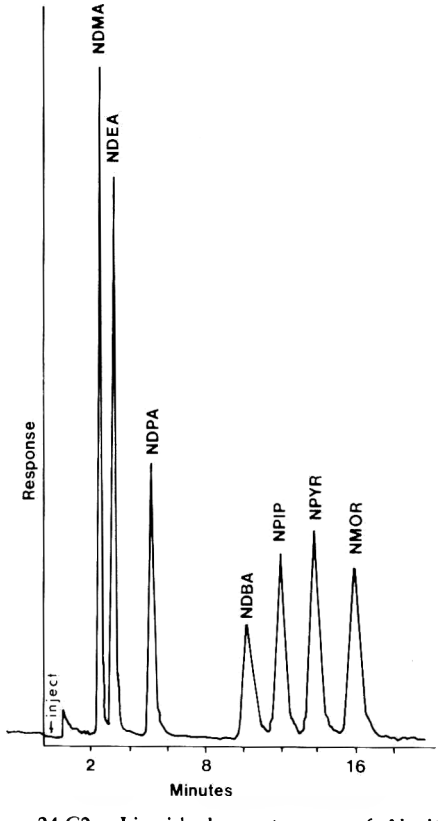


Figure 24:C2. Liquid chromatogram of *N*-nitrosamines mixed standard solution: 1.50 ng NDMA; 1.58 ng NDEA; 1.46 ng NDPA; 1.59 ng NDBA; 1.64 ng NPIP; 1.67 ng NPYR; 1.80 ng NMOR.

Table 1. Collaborative results for dimethylnitrosamine (ppb)

Coll.	Sample 1	2	3	4	5	6	7	8	9
1	1.03	4.56	7.77	10.2	12.1	16.2	1.00	1.69	1.97
	0.56	5.30	7.08	10.1	13.1	16.2	0.98	1.83	1.99
					13.5		0.91	1.56	
					14.1			1.42	
2	0.4	5.5	6.7	10.4	14.2	15.1	0.82	2.11	3.3
	0.40	4.8	7.8	9.6	12.4	15.3	0.8	1.4	2.0
					11.3		0.7	1.3	
					12.3			1.3	
3	0.67	5.31	7.74	10.8	14.9	24.5	0.98	1.96	2.38
	0.57	5.03	6.99	9.88	13.0	16.6	0.80	2.37	2.79
					13.0		1.28	2.53	
					12.4			2.47	
4	0.61	4.53	6.23	8.87	9.59	14.7	0.90	1.43	2.28
	0.41	4.77	6.61	8.64	11.4	15.1	0.61	1.39	2.08
					11.4		0.88	1.43	
					11.9			1.43	
5	1.12	7.09	9.28	10.9	15.1	20.4	1.12	2.00	2.68
	1.60	6.25	8.47	12.0	14.6	18.4	1.14	2.25	2.45
					16.3		0.98	2.53	
					14.4			2.11	
6	1.7	5.1	7.1	8.7	11.1	14.2	1.7	2.1	2.8
	1.6	5.3	7.5	9.0	11.4	14.5	1.4	4.0	3.3
					11.8		3.1	3.5	
					11.4			3.5	
7[a]	21.4	8.09	7.59	14.2	24.1	20.6	1.27	2.25	2.34
	—[b]	6.66	9.09	13.1	14.4	21.0	—[b]	1.68	3.16
					20.9		1.30	1.46	
					20.4			2.67	
8	0.34	3.53	5.58	8.68	8.37	14.4	0.74	1.36	2.32
	0.42	4.77	6.21	8.90	11.1	13.2	0.83	1.40	2.40
					10.8		0.82	1.53	
					10.8			1.43	
9[a]	2.40	6.05	6.83	8.83	15.0	20.9	1.75	2.39	1.91
	1.48	5.40	6.82	8.49	9.56	12.7	0.83	2.24	2.24
					15.7		1.19	2.61	
					16.1			2.11	
All labs:									
$\bar{x}$, ppb	0.96	5.45	7.30	10.07	13.44	16.89	1.11	2.02	2.47
s	0.62	1.06	0.97	1.63	3.22	3.29	0.50	0.67	0.45
CV, %	64.6	19.4	13.3	16.2	24.0	19.5	45.0	33.2	18.2
Outliers excl.:									
$\bar{x}$, ppb	0.82	5.13	7.22	9.76	12.42	16.34	1.07	1.98	2.48
s	0.50	0.83	0.97	1.03	1.77	2.98	0.53	0.72	0.44
CV, %	61.0	16.2	13.4	10.6	14.3	18.2	49.5	36.4	17.7

[a] Data not included in statistical evaluation.
[b] Sample lost.

NPIP, NPYR, and NMOR. DBNA had a lower correlation coefficient, which may be caused by phenomena observed in our laboratories previously. DBNA is influenced to a larger extent by temperature fluctuations and appears to reflux with consequently lower recoveries. For DMNA, DENA, NPIP, NPYR, and NMOR, collaborators with a correlation coefficient less than 0.95 were classified as performance outliers. For DBNA, laboratories with correlation coefficients less than 0.90 were classified as performance outliers.

(2) The mean ratio technique appears to be a technique that is applicable to chemical conditions/procedures that may not go to complete equilibrium, such as low partition coefficients between solvents or a distillation at micro levels. It makes it possible to evaluate pooled data at

Table 2. Collaborative results for diethylnitrosamine (ppb)

Coll.	Sample 1	2	3	4	5	6	7	8	9
1	1.09	4.61	6.99	9.38	11.6	16.1	0.87	0.96	1.55
	1.43	5.77	7.59	9.86	12.3	15.7	1.53	ND	ND
					12.2		ND	ND	
					12.1			ND	
2	ND[a]	4.6	5.5	8.8	12.0	12.8	ND	ND	ND
	ND	3.8	6.5	8.0	11.0	13.0	ND	ND	ND
					10.4				
					11.2				
3	ND	5.23	6.91	9.64	12.8	15.8	ND	ND	ND
	ND	4.70	6.67	9.66	12.8	17.1	ND	ND	ND
					12.5		ND	ND	
					12.1				
4	0.43	4.72	6.21	9.43	10.6	15.1	ND	ND	ND
	ND	4.58	6.32	8.92	11.6	15.5	ND	ND	ND
					11.6		ND	ND	
					12.0			ND	
5	ND	5.40	7.09	9.37	12.2	16.8	ND	ND	ND
	ND	4.85	7.05	10.90	13.1	16.6	ND	ND	ND
					13.7		ND	ND	
					12.7			ND	
6	0.9	4.8	6.8	8.8	11.5	14.2	1.0	1.0	0.7
	0.7	4.6	6.7	8.8	11.3	14.6	0.6	1.4	0.8
					10.9		1.6	1.2	
					11.4			1.0	
7	ND	5.57	6.20	11.5	16.4	17.7	ND	ND	ND
	—[b]	5.56	7.94	12.0	12.1	10.8	—[b]	ND	ND
					16.9		ND	ND	
					12.9			ND	
8	ND	3.78	5.86	8.14	7.88	14.2	ND	ND	ND
	ND	4.70	6.43	8.65	11.20	13.2	ND	ND	ND
					10.40				
					10.40				
9[c]	ND	4.05	5.68	7.67	12.6	17.2	ND	ND	ND
	ND	3.55	4.99	6.34	7.78	10.1	ND	ND	ND
					11.8				
					10.8				
All labs:									
$\bar{x}$		4.72	6.52	9.21	11.85	15.36			
s		0.64	0.73	1.35	1.70	2.35			
CV, %		13.6	11.2	14.6	14.3	15.3			
Outliers excl.:									
$\bar{x}$		4.83	6.67	9.49	11.99	15.58			
s		0.57	0.61	1.13	1.63	2.03			
CV, %		11.8	9.1	11.9	13.7	13.0			

[a] Not detected.
[b] Sample lost.
[c] Data not included in statistical evaluation.

different levels. The mean is determined at each level to generate a ratio table. Each data point is divided by the mean at each level. The mean ratio for each collaborator is determined (Table 9). Ratios of less than 0.80 or more than 1.20 were classified as performance outliers. The mean ratio would correlate to average individual recoveries of approximately 72–108%.

(3) The coefficient of variation for each nitrosamine and each collaborator was determined (Table 10). Coefficients of variation higher than 13.0% were classified as outliers for DMNA. The value of 12.5% was used for DENA, NPIP, NPYR, and NMOR; 14.0% was used for DBNA. The value for DPNA, because it is an internal standard, should be less than 9.0%.

Table 3. Collaborative results for dibutylnitrosamine (ppb)

	Sample								
Coll.	1	2	3	4	5	6	7	8	9
1	ND[a]	3.89	5.89	8.79	10.8	16.5	ND	ND	ND
	ND	4.38	5.85	10.10	12.2	13.6	ND	ND	ND
					11.4		ND	ND	
					11.6				
2	ND	3.3	4.6	7.5	11.7	11.4	ND	ND	ND
	ND	3.7	5.9	6.5	10.1	11.2	ND	ND	ND
					7.6			ND	
					8.4			ND	
3	ND	4.36	6.09	9.36	12.7	15.7	ND	ND	ND
	ND	4.68	6.43	9.88	12.5	16.7	ND	ND	ND
					19.6		ND	ND	
					11.8			ND	
4	ND	3.38	4.28	7.67	7.01	11.2	ND	ND	ND
	ND	4.22	5.89	7.88	9.92	13.8	ND	ND	ND
					10.0				
					11.5				
5	ND	4.21	5.11	7.95	9.71	13.4	ND	ND	ND
	ND	3.86	5.28	7.80	10.9	13.5	ND	ND	ND
					13.2				
					10.0				
6[b]	ND	2.3	3.2	6.1	4.8	6.5	ND	ND	ND
	ND	3.5	3.9	8.1	9.4	12.9	ND	ND	ND
					9.2				
					9.7				
7	ND	4.30	5.37	8.66	11.1	13.0	ND	ND	ND
	—[c]	4.46	5.95	9.68	9.68	17.4	—[c]	ND	ND
					10.8		ND	ND	
					9.27				
8	ND	3.60	6.29	8.68	8.20	13.0	ND	ND	ND
	ND	4.91	6.01	7.64	9.73	12.1	ND	ND	ND
					10.0			ND	ND
					11.3				ND
9[b]	ND	2.96	3.90	6.57	7.55	11.3	ND	ND	ND
	ND	2.42	3.22	5.00	5.78	10.2	ND	ND	ND
					10.9			ND	
					9.06				
All labs:									
$\bar{x}$		3.80	5.18	7.99	10.25	12.97			
s		0.73	1.06	1.36	2.46	2.62			
CV, %		19.2	20.5	17.0	24.0	20.2			
Outliers excl.:									
$\bar{x}$		4.09	5.64	8.44	10.81	13.75			
s		0.48	0.63	1.05	2.29	2.08			
CV, %		11.7	11.2	12.4	21.2	15.1			

[a] Not detected.
[b] Data not included in statistical evaluation.
[c] Sample lost.

Table 4. Collaborative results for nitrosopiperidine (ppb)

	Sample								
Coll.	1	2	3	4	5	6	7	8	9
1	ND[a]	3.89	5.96	8.79	10.6	16.5	0.80	1.11	0.80
	ND	4.56	6.23	9.86	12.1	14.0	0.78	0.98	0.73
					11.6		0.71	0.78	
					11.6			0.90	
2	ND	4.4	5.6	8.6	13.5	13.3	ND	ND	ND
	ND	4.8	7.2	8.8	10.5	14.0	ND	2.0	0.9
					10.0		1.50	2.6	
					10.9			1.5	
3	ND	4.77	6.79	9.82	12.8	16.4	ND	0.42	ND
	ND	4.84	6.48	9.65	12.2	16.5	ND	1.19	0.46
					11.9		0.37	0.94	
					11.8			0.92	
4	ND	4.41	6.01	8.96	11.0	14.1	0.57	0.48	ND
	ND	4.74	6.28	9.31	11.4	15.6	ND	ND	ND
					11.5				
					12.3				
5	ND	5.11	6.96	9.38	12.1	16.4	0.72	1.26	0.95
	ND	5.21	7.05	10.20	12.9	16.4	0.81	1.53	0.88
					13.5		0.88	1.59	
					12.5			1.65	
6	ND	3.7	5.4	7.8	9.0	11.0	ND	1.0	1.0
	0.5	4.3	5.5	8.6	10.4	13.6	0.7	1.2	0.6
					9.8		0.6	1.0	
					10.0			0.8	
7[b]	ND	4.96	6.24	9.55	12.4	15.4	ND	ND	ND
	—[c]	5.25	7.00	9.92	10.4	20.5	—[c]	ND	ND
					13.1			ND	
					12.4				
8	ND	4.10	6.62	8.41	8.17	14.6	ND	ND	ND
	ND	5.10	6.28	9.09	11.8	13.6	ND	ND	ND
					10.6				
					11.2				
9	ND	3.41	4.63	7.60	8.93	13.7	ND	ND	ND
	ND	2.48	3.81	5.42	6.71	9.63	ND	ND	ND
					11.30		ND	ND	
					9.49				
All labs:									
$\bar{x}$		4.45	6.11	8.88	11.18	14.74			
s		0.72	0.88	1.12	1.51	2.38			
CV, %		16.2	14.4	12.6	13.5	16.1			
Outliers excl.:									
$\bar{x}$		4.57	6.31	9.09	11.34	14.72			
s		0.46	0.58	0.66	1.27	1.65			
CV, %		10.1	9.2	7.3	11.2	11.2			

[a] Not detected.
[b] Data not included in statistical evaluation.
[c] Sample lost.

Table 5. Collaborative results for nitrosopyrrolidine (ppb)

	Sample								
Coll.	1	2	3	4	5	6	7	8	9
1	4.08	5.90	9.66	12.8	14.0	18.9	9.68	17.4	13.2
	3.92	8.25	9.67	12.8	14.1	18.2	10.5	18.0	14.1
					15.9		11.2	17.4	
					16.0			16.2	
2	3.0	7.4	8.6	10.4	15.0	18.0	9.86	21.2	10.1
	3.11	7.6	9.5	12.1	13.3	18.8	8.5	18.0	16.6
					12.8		9.2	17.2	
					13.8			15.8	
3	2.67	8.01	9.75	12.3	15.2	18.7	8.80	21.7	16.3
	2.68	7.66	9.11	12.0	14.8	18.1	9.55	21.6	15.9
					14.1		10.60	23.0	
					13.7			20.2	
4	3.32	7.33	8.87	12.1	14.0	17.8	10.7	18.0	17.3
	2.86	7.37	9.38	11.8	14.0	17.8	10.6	18.1	15.6
					14.4		10.5	17.7	
					15.2			19.1	
5	3.28	8.37	10.5	12.4	15.1	19.4	10.7	20.7	18.2
	3.75	7.85	10.0	13.4	15.6	19.2	10.4	22.1	16.6
					17.5		11.2	22.3	
					16.1			21.2	
6	3.2	5.6	7.7	11.0	12.2	14.7	10.4	15.8	15.5
	3.0	7.1	7.5	11.0	14.0	16.8	9.7	14.7	12.6
					11.6		8.7	14.5	
					12.5			14.3	
7	3.30	7.66	8.90	10.6	15.3	17.2	10.5	13.2	17.9
	—[a]	8.21	8.73	12.8	13.0	23.2	—[a]	18.7	17.2
					15.8		11.4	16.0	
					14.9			21.4	
8	3.47	6.66	9.40	10.8	11.3	16.2	9.82	15.8	18.0
	3.12	7.68	8.19	12.5	14.3	15.3	9.01	15.5	14.5
					12.3		10.30	15.3	
					14.6			15.8	
9[b]	2.34	5.66	6.11	9.31	9.90	15.3	11.10	17.8	10.8
	2.35	4.47	6.43	7.53	8.14	11.4	7.22	13.8	12.2
					14.8		6.80	14.3	
					11.5			12.9	
All labs:									
$\bar{x}$	3.14	7.16	8.78	11.54	13.91	17.5	9.88	17.69	15.14
s	0.49	1.08	1.19	1.45	1.86	2.45	1.17	2.87	2.49
CV, %	15.6	15.1	13.6	12.6	13.4	14.0	11.8	16.2	16.4
Outliers excl.:									
$\bar{x}$	3.25	7.42	9.09	11.93	14.25	18.02	10.08	18.06	15.60
s	0.42	0.79	0.82	0.91	1.40	1.92	0.83	2.75	2.22
CV, %	12.8	10.6	8.9	7.6	9.8	10.7	10.1	15.2	14.3

[a] Sample lost.
[b] Data not included in statistical evaluation.

Table 6. Collaborative results for nitrosomorpholine (ppb)

	Sample								
Coll.	1	2	3	4	5	6	7	8	9
1	ND[a]	3.70	6.19	8.98	11.2	16.0	ND	ND	ND
	ND	4.69	6.47	9.70	12.0	14.6	ND	ND	ND
					11.7		ND	ND	ND
					12.0			ND	
2	ND	4.4	6.2	8.6	12.0	14.0	ND	ND	ND
	ND	5.5	6.6	8.7	10.7	13.7	ND	ND	ND
					12.5		ND	ND	
					12.3			ND	
3	ND	4.36	6.34	9.80	12.8	17.2	ND	ND	ND
	ND	4.75	6.77	9.96	13.2	17.7	ND	ND	ND
					11.7		ND	ND	
					11.4			ND	
4	ND	4.69	6.12	10.1	10.7	15.1	0.48	ND	ND
	ND	4.86	6.71	8.98	11.5	15.9	ND	ND	ND
					12.3		ND	ND	
					12.1			ND	
5	ND	5.39	7.04	9.39	12.7	16.7	ND	ND	ND
	ND	4.62	7.23	9.89	12.3	17.1	ND	ND	ND
					14.4		ND	ND	
					12.2				ND
6	ND	3.3	5.5	9.1	9.5	12.7	ND	ND	ND
	ND	4.3	5.5	8.6	11.1	14.1	ND	ND	ND
					10.2		ND	ND	
					10.6				
7	ND	5.10	6.35	10.2	12.7	15.7	ND	ND	ND
	—[b]	4.87	6.26	10.4	11.2	20.1	—[b]	ND	ND
					13.2				
					12.2				
8	ND	4.38	6.95	9.01	8.86	14.9	ND	ND	ND
	ND	4.92	6.36	9.19	11.7	15.9	ND	ND	ND
					11.1				
					11.6				
9[c]	ND	3.59	5.27	8.41	8.94	13.5	ND	ND	ND
	ND	3.25	5.29	7.17	8.34	11.8	ND	ND	ND
					13.5		ND	ND	
					9.30				
All labs:									
$\bar{x}$		4.48	6.29	9.23	11.55	15.37			
s		0.65	0.58	0.80	1.36	1.99			
CV, %		14.1	9.2	8.7	11.8	12.9			
Outliers excl.:									
$\bar{x}$		4.61	6.41	9.41	11.74	15.71			
s		0.56	0.48	0.60	1.10	1.81			
CV, %		12.1	7.5	6.4	9.4	11.5			

[a] Not detected.
[b] Sample lost.
[c] Data not included in statistical evaluation.

Table 7. Recovery of dipropylnitrosamine internal quality control standard (%)

	Sample								
Coll.	1	2	3	4	5	6	7	8	9
1	94	88	99	95	93	114	90	93	79
	92	89	88	89	88	90	83	89	83
					93		90	89	
					94			82	
2	87	86	89	85	92	82	83	82	84
	91	89	89	86	94	85	90	90	84
					82		78	83	
					83			78	
3	97	102	95	101	97	95	94	99	102
	100	107	108	107	104	108	104	105	97
					96		106	106	
					97			108	
4	90	89	87	94	84	90	92	85	94
	90	88	90	91	90	88	91	86	91
					85		94	89	
					93			90	
5	111	116	116	106	112	112	104	106	96
	99	103	103	104	103	96	98	112	91
					111		99	109	
					103			102	
6	101	97	103	103	103	96	108	99	91
	98	99	96	91	98	94	102	96	102
					97		95	103	
					106			101	
7	88	93	84	96	100	89	91	95	81
	—[a]	84	86	89	91	86	—[a]	101	108
					110		88	86	
					91			105	
8	72	73	80	78	77	76	79	76	86
	80	80	78	76	78	81	83	80	83
					80		80	89	
					90			88	
9	76	82	78	81	81	90	97	89	89
	78	92	99	98	87	90	99	97	81
					110		97	96	
					110			100	
$\bar{x}$					92.8				
s					9.57				
CV, %					10.3				

[a] Sample lost.

Table 8. Least squares linear regression correlation coefficients for nitrosamine-fortified samples

Coll.	DMNA	DENA	DBNA	NPIP	NPYR	NMOR
1	0.9941	0.9963	0.9887	0.9912	0.9857	0.9958
2	0.9882	0.9903	0.9677	0.9808	0.9879	0.9899
3	0.9549	0.9980	0.9472	0.9984	0.9959	0.9937
4	0.9934	0.9970	0.9646	0.9961	0.9978	0.9946
5	0.9932	0.9966	0.9838	0.9979	0.9935	0.9950
6	0.9977	0.9989	0.8779[a]	0.9856	0.9826	0.9905
7	0.9211[b]	0.9689	0.9657	0.9567	0.9504	0.9750
8	0.9846	0.9812	0.9800	0.9799	0.9730	0.9847
9	0.9049[b]	0.9292[b]	0.9510	0.9463[b]	0.9143[b]	0.9603

Data not included in statistical evaluation:
[a] Correlation coefficient less than 0.90, DBNA only.
[b] Correlation coefficient less than 0.95.

Table 9. Tabulation of mean ratios for each nitrosamine to grand mean

Coll.	DMNA	DENA	DBNA	NPIP	NPYR	NMOR
1	0.991	1.035	1.064	0.996	1.026	0.997
2	1.007	0.890	0.863	0.995	0.962	0.983
3	1.076	1.035	1.188	1.073	1.033	1.043
4	0.895	0.960	0.905	1.004	1.009	1.003
5	1.194	1.070	0.980	1.123	1.115	1.083
6	0.983	0.953	0.739[a]	0.867	0.885	0.873
7	1.369[a]	1.186	1.056	1.114	1.033	1.066
8	0.852	0.871	1.258[a]	0.965	0.941	0.977
9	1.102	0.832	0.715[a]	0.738[a]	0.757[a]	0.817

[a] Data not included in statistical evaluation because ratio is below 0.800 or above 1.200.

Table 10. Laboratory coefficients of variation (%) for nitrosamines collaboratively studied

Coll.	DMNA	DENA	DPNA	DBNA	NPIP	NPYR	NMOR
1	10.3	6.6	7.6	7.2	7.4	10.6	6.6
2	12.6	7.8	5.0	13.0	10.0	10.5	9.4
3	12.9	3.6	4.8	17.0[a]	3.1	10.8	5.7
4	5.2	3.4	3.2	13.9	4.1	4.5	5.0
5	8.4	4.8	6.4	8.7	3.4	5.7	6.2
6	12.6	3.8	4.4	25.7[a]	7.0	8.7	8.1
7	19.5[b]	12.1	8.8	10.6	12.7[c]	11.7	8.2
8	11.0	8.6	5.9	12.6	10.3	9.8	8.4
9	19.5[b]	18.6[c]	10.6[d]	18.9[a]	19.6[c]	17.5[c]	14.5[c]

[a] Data not included; CV more than 14.0.
[b] Data not included; CV more than 13.0.
[c] Data not included; CV more than 12.5.
[d] Data for all analyses not included in statistical evaluation because CV for quality control standard is more than 9.0.

Table 11. Compilation of laboratory outliers by all techniques

Statistic	DMNA	DENA	DBNA	NPIP	NPYR	NMOR	DPNA
r	7,9	9	6	9	9		
Mean ratio	7		6,8,9	9	9		
CV, %	7,9	9	3,6,9	7,9	9	9	
DPNA							9
Youden	7,8	7,9	3,6,9	5,9	6,9	9	

Table 12. Summary of statistical data

Statistic[a]	DMNA	DENA	DBNA	NPIP	NPYR	NMOR
			All Laboratories			
Range, ppb	3–16	4–16	4–17	4–15	3–18	4–16
CV_0	15.6	12.6	19.7	11.8	12.0	9.6
CV_x	23.7	16.7	25.6	16.7	17.8	13.0
CV_x/CV_0	1.52	1.32	1.30	1.42	1.48	1.35
			Outlier Laboratories Excluded (Performance Standards)			
CV_0	10.8	8.5	10.4	8.5	8.7	7.8
CV_x	16.4	12.0	13.6	10.8	11.2	10.3
CV_b	8.7	6.0	6.2	4.7	5.0	4.8
CV_x/CV_0	1.52	1.40	1.31	1.26	1.28	1.31
			Outlier Laboratories Excluded (Youden)			
CV_0	10.7	6.5	12.3	8.7	7.5	7.4
CV_x	14.5	10.1	14.3	11.4	8.9	9.7
CV_x/CV_0	1.36	1.55	1.16	1.31	1.19	1.31

[a] CV_0 = coefficient of variation for repeatability; CV_x = coefficient of variation for reproducibility; CV_b = coefficient of variation for bias.

Table 13. Sample fortification level (ppb)

Sample[a]	DMNA	DENA	DBNA	NPIP	NPYR	NMOR
1	0	0	0	0	0	0
2	5.05	5.15	4.84	4.96	4.89	5.22
3	7.07	7.21	6.78	6.94	6.84	7.31
4	10.1	10.3	9.68	9.92	9.78	10.4
5	13.1	13.4	12.7	12.9	12.7	13.7
6	17.2	17.3	16.5	16.8	16.6	17.8
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0

[a] Nitrosamines reported in Samples 1, 7, 8, and 9 were natural contaminants of the cooking process.

Table 14. Nitrosamine recoveries (%) from fortified samples

Sample	DMNA	DENA	DBNA	NPIP	NPYR	NMOR
			All Laboratories			
1	117.1[a]	—	—	—	92.9[a]	—
2	92.8	91.6	78.5	89.7	86.4	85.8
3	92.5	90.4	76.4	88.0	85.8	86.0
4	92.2	89.4	82.5	89.5	87.6	88.8
5	96.6	88.4	80.7	86.7	86.7	84.3
6	93.7	88.8	78.6	87.7	87.6	86.3
$\bar{x}$	93.6	89.7	79.3	88.3	86.8	86.2
			Outliers Excluded			
1	100.0[a]	—	—	—	96.0[a]	—
2	87.3	93.8	84.5	92.1	89.7	88.3
3	91.5	92.5	83.2	90.9	88.9	87.7
4	89.4	92.1	87.2	91.6	90.6	90.5
5	89.2	89.5	85.1	87.9	88.6	85.7
6	90.7	90.0	83.3	87.6	90.2	88.3
$\bar{x}$	89.6	91.6	84.7	90.0	89.6	88.1

[a] Not included in the determination of the average.

Table 15. Pooled tabulation of coefficients of variation (%) for NPYR

No. of Labs	No. of Samples	CV_x	CV_0	CV_b
9	9	14.7	9.5	8.0
9	11	14.8	9.3	8.1
8	9	11.2	8.7	5.0
7	9	11.2	8.0	5.5
7	11	11.8	7.6	6.4

The performance outliers of the collaborative study are not statistical outliers. There are several specific data points that could be considered statistical outliers but no effort was made to identify them. The number of samples and the levels were chosen to describe the method on each side of the level of interest and to establish standards of performance for the method. The results that were not included in the statistical evaluation were eliminated on the basis of higher variability or lower recovery than is acceptable for regulatory analysis.

Table 11 is a tabulation of the performance outliers and outliers by Youden's technique and confirms the need for an internal quality control standard. The correct internal quality control standard provides an immediate check on the acceptability of each analysis. NDPA is not the ideal internal standard because 3 laboratories had unacceptable data for one or more of the nitrosamines but only one laboratory had performance outliers for every nitrosamine. The improvement in the CV values by the elimination of performance outliers can be observed in Tables 1–6. The averages, standard deviations, and coefficients of variation for the acceptable laboratories were added to the tables.

Pooled CV values for repeatability and reproducibility were determined using the technique of Steiner (3) for all samples and with outliers excluded. In addition, a CV value for bias (per-

formance outlier excluded only) was determined using Youden's (3) formula, $s_d^2 = 2s_b^2 + s_r^2$. They are tabulated in Table 12.

Youden's technique would have eliminated more data and in 2 cases would have eliminated 2 different laboratories (Table 11). The 3 performance techniques provide a perspective that is not easily available if only Youden's technique is used.

A discussion of bias would not be complete without discussing the recoveries of the fortified samples. The recoveries were calculated from the fortified values for the nitrosamines listed in Table 13. For DMNA and NPYR, the regressed values of 0.82 and 3.385 were added to the fortified values to determine recovery. The average recoveries by level for each nitrosamine and the grand averages are tabulated in Table 14 for all laboratories and for the acceptable laboratories. Higher recoveries were obtained for the acceptable laboratories for all nitrosamines except DMNA. The recovery of all laboratories for DMNA was high because one laboratory reported unusually high values. The recoveries at these levels and the bias CV values of the study are excellent and demonstrate the ruggedness of the procedure and its acceptability for regulatory analysis.

Table 15 is a tabulation of the pooled CV values for NPYR to enable a comparison of the pooled values for all the data, the data with the performance outliers of the study, and the data with a more vigorous performance standard. Also, for the 2 additional sets of data, the quadruplicate samples were treated as 2 pairs of additional samples. If we did not wish to describe the method rigorously, that is, to define the results that would be obtained with proficient laboratories, the CV values for the data from all the laboratories would be acceptable. The method that was studied was straightforward, and it should not be difficult for any laboratory to achieve the minimum standards described.

Recommendation

It is recommended that the mineral oil vacuum distillation–TEA method for determining nitrosamines in cooked bacon be adopted official first action.

The performance standards to be used to determine proficiency are as follows: a correlation coefficient, least squares linear regression on a minimum of 6 sample duplicate analyses, >0.95 for DMNA, DENA, NPIP, NPYR, and NMOR and >0.90 for DBNA. The average recovery for a fortified sample, minimum of 12 values, should be 72–108%. The coefficient of variation for recoveries should be <9.0 for DPNA, <14.0 for DBNA, 12.5 for DENA, NPIP, NPYR, and NMOR, and <13.0% for DMNA.

Acknowledgments

We acknowledge and thank Walter Fiddler, USDA, Wyndmoor, PA; Thomas Fazio, FDA, Washington, DC; William Brown, ABC Laboratory, Gainesville, FL; and Burt Levy, USDA, Washington, DC, for assistance in planning the study; and Michael W. O'Donnell, FDA, Washington, DC, for assistance and critique of the statistical evaluation.

We also thank the following collaborators: M. Benson, Hormel; W. Fiddler, U.S. Dept of Agriculture; M. Grabber, Armour & Co.; H. Herring, Wilson and Co.; K. Hill, Peter Eckrich; Keith A. Meyer, Swift & Co.; Liston Legette, U.S. Dept of Agriculture; Michael C. Robach, Monsanto; and Martin Wolfe, Thermo Electron Co.

References

(1) Greenfield, E. L., Smith, W., & Malanoski, A. J. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1316–1318
(2) Malanoski, A. J. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1333–1338
(3) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA

Analyst Performance Standards: Determination for and from Collaborative Studies

ANTHONY J. MALANOSKI
Food Safety and Inspection Service, Chemistry Division, Washington, DC 20250

With the increased emphasis on quality assurance in all phases of regulatory analysis, it has become necessary to establish standards of performance for the analyst. The technique discussed is one that should be considered for use and the performance standard should become an integral part of the method.

The emphasis on quality assurance programs in recent years has created a need to establish performance standards that analysts must meet to demonstrate their proficiency. The recent proposal of the Food Safety and Inspection Service to accredit laboratories to perform analyses has emphasized that efforts should be made to develop techniques for establishing performance standards. The primary source proposed to establish standards is the collaborative study as defined and used by the Association of Official Analytical Chemists. These guidelines address collaborative studies in general but do not address the problems that to some extent are unique as the quantity to be determined approaches zero.

Two recent publications, one by the ACS Committee on Environmental Improvement (Guidelines for Data Acquisitions and Data Quality Evaluation in Environmental Chemistry) (1) and the second by Horwitz et al. (2), cover several aspects of performance but do not discuss performance standards that analysts should meet before their data are reported for use. That all analysts are competent cannot be assumed, especially with the advent of residue analytical chemistry, because of the higher level of proficiency and experience necessary to perform residue analysis. The primary purpose of the collaborative study is to evaluate the method, based on the assumption that only competent, experienced analysts who were proficient in the analysis would participate. If this assumption were true, then the study could be limited to fewer samples and outliers could be eliminated by means of standard statistical tests. Because of the complexity of the analytical procedures for residue analyses, the levels (microgram and nanogram quantities), and the need for a higher level of training and experience, minimum performance standards should be established for and from the collaborative study. The performance standards established from the collaborative study then become a specification of the method.

To establish valid and meaningful performance standards does require that each participating laboratory perform more analyses. The additional analyses are necessary to estimate individual proficiency. It may be necessary for the participating laboratories to complete a familiarization phase before the study is initiated. If performance standards are established and analysts' data are eliminated on the basis of performance, then no test to determine statistical outliers should be performed. If the method as studied does not meet regulatory requirements, then the method should be critically reviewed again to determine if all of the critical points had been identified, with performance standards for these points listed before the collaborative study was initiated. A technique preferred in our program is a preliminary 3-laboratory validation study, to verify the adequacy and completeness of the method. The 3-laboratory study also is used to establish preliminary performance standards for the collaborative study.

A collaborative study that is to be used to evaluate both the method and the performance of each analyst is more complex and may require minor changes in the statistical analysis of the data. A necessary attribute for reproducible analysis is that the analyst perform all critical manipulations as identically as possible, which can only be determined when a larger number of samples is analyzed. When different levels are used, it is necessary to normalize the data so all data can be pooled to evaluate individual performance. To normalize data, the mean value of all reported values has been used by FSIS. The mean is either subtracted or used as a divisor to normalize the data. The determination of which techniques should be used is made on the basis of whether the standard deviation or the coefficient of variation (CV) is more constant over the range of the levels of interest.

The dominant variance determines the technique that should be used in obtaining pooled

Received June 18, 1981. Resubmitted February 8, 1982. Accepted June 17, 1982.

0004-5756/82/6506-1333-06$01.00

variances. In this paper, we are primarily concerned with analysis at residue levels with a range in the levels of interest from x to 4x or 5x. The CV value is the more consistent value. The transformation proposed is to divide each individual value by the mean value of all analysts for each level. The use of subtraction to code values has long been used to reduce mathematical manipulations before the development of computers and is described in all standard statistical texts. Division by the mean value was suggested from the statistical analyses of recoveries from fortified samples. The technique when used for analysis of both fortified samples and samples with incurred residues permits the use of all values in the evaluation of individual CV values (CV_a). To date, in the studies conducted by FSIS when the mean ratio technique has been used to evaluate data, the CV value of the transformed data and the CV value of the raw data are equivalent. Therefore the transformation of different levels into the ratios for each participant can be treated to statistical analysis and can be used to establish a standard of performance.

The second technique that may used to establish performance standards when fortified samples are included is the least squares linear regression. The correlation coefficient, the slope, and the intercept can all be used to evaluate individual laboratory performance.

The use of a standard of performance established from the collaborative study should then be included in the analytical procedure as a specification similar to instrument specifications. It is as important a requirement as specifying instrument parameters to achieve method reproducibility. However, standards of performance must be established by a chemist familiar with the analytical procedure, in other words, standards should be established by chemists. To establish proficiency standards, it is necessary to obtain more data points from each laboratory. Suggested minimum collaborative study requirements are as follows:

Laboratories	3	4	5	6	≥7
Fortified samples	6	6	6	5	5
Incurred samples	2	2	2	2	2
Replicates	5	4	3	2	2
Total values	180	192	180	120	≥140

The fortified levels (nominal) should be 0.0x, 0.5x, 0.7x, 1x, 1.5x, and 2x with 2 incurred levels in the range of 0.7x–1.5x (x is the tolerance or guideline level).

Establishing performance standards can be demonstrated most simply by an example from a recent nitrosamine collaborative study (3). The discussion will be limited to nitrosopyrrolidine (NPYR). Nine laboratories analyzed a total of 9 samples in either duplicate, triplicate, or quadruplicate. The fortified levels were 0, 4.89, 6.84, 9.78, 12.7, and 16.6 ppb with incurred levels of approximately 10, 15, and 18 ppb.

Raw data of Table 1 were initially evaluated by means of the least squares linear regression. Table 2 is a tabulation of the regression equations, correlation coefficients, and approximate recovery. In the collaborative study as reported, only the correlation coefficient was used. A minimum performance standard of 0.95 was used. However, when both the intercept and correlation coefficient are evaluated jointly, the correlation coefficient standard could be a minimum of 0.97. The intercept of the linear regression equation is an estimate of the nitrosopyrrolidine present in the original sample. Laboratories 7 and 9 are sufficiently different to identify them as suspect performance outliers.

The mean value, standard deviation, coefficient of variation, and mean recovery were then calculated to determine homogeneity of the data. The value of 3.385 ppb NPYR was determined from the regressed data (excluding Laboratories 7 and 9) as the NPYR present in the sample used for fortification. To determine the recovery, the estimated value present was added to each fortified value. The recoveries with a range of 85.3–92.7% appear to be homogeneous and are acceptable for the levels studied. The standard deviations from 0.49 to 2.87 ppb strongly suggest that the data should not be used to determine a pooled standard deviation. The CV values, however, appear to be homogeneous (11.8 to 16.4) and could be considered acceptable without further evaluation.

To establish an analyst performance standard, additional information is necessary. Because the CV values were more constant, a transformation of the data was generated by dividing each individual value by the mean level. The transformed data are given in Table 3. The mean, standard deviation, and CV value for each level and laboratory were determined. A comparison of CV values by level in Tables 1 and 2 demonstrate their equivalence. The CV values for laboratories ranged from 4.3 to 17.1%. The 17.1% value for Laboratory 9 is unacceptably high and identifies the laboratory as a performance outlier. Laboratory 7 with a value of 11.8% was a suspect outlier from the regression analysis. The 2 indicators together identify Laboratory 7 as a pos-

Table 1. Collaborative results for nitrosopyrrolidine (ppb)

	Sample								
Coll.	1	2	3	4	5	6	7	8	9
1	4.08	5.90	9.66	12.8	14.0	18.9	9.68	17.4	13.2
	3.92	8.25	9.67	12.8	14.1	18.2	10.5	18.0	14.1
					15.9		11.2	17.4	
					16.0			16.2	
2	3.0	7.4	8.6	10.4	15.0	18.0	9.86	21.2	10.1
	3.11	7.6	9.5	12.1	13.3	18.8	8.5	18.0	16.6
					12.8		9.2	17.2	
					13.8			15.8	
3	2.67	8.01	9.75	12.3	15.2	18.7	8.80	21.7	16.3
	2.68	7.66	9.11	12.0	14.8	18.1	9.55	21.6	15.9
					14.1		10.60	23.0	
					13.7			20.2	
4	3.32	7.33	8.87	12.1	14.0	17.8	10.7	18.0	17.3
	2.86	7.37	9.38	11.8	14.0	17.8	10.6	18.1	15.6
					14.4		10.5	17.7	
					15.2			19.1	
5	3.28	8.37	10.5	12.4	15.1	19.4	10.7	20.7	18.2
	3.75	7.85	10.0	13.4	15.6	19.2	10.4	22.1	16.6
					17.5		11.2	22.3	
					16.1			21.2	
6	3.2	5.6	7.7	11.0	12.2	14.7	10.4	15.8	15.5
	3.0	7.1	7.5	11.0	14.0	16.8	9.7	14.7	12.6
					11.6		8.7	14.5	
					12.5			14.3	
7	3.30	7.66	8.90	10.6	15.3	17.2	10.5	13.2	17.9
	—[a]	8.21	8.73	12.8	13.0	23.2	—[a]	18.7	17.2
					15.8		11.4	16.0	
					14.9			21.4	
8	3.47	6.66	9.40	10.8	11.3	16.2	9.82	15.8	18.0
	3.12	7.68	8.19	12.5	14.3	15.3	9.01	15.5	14.5
					12.3		10.30	15.3	
					14.6			15.8	
9	2.34	5.66	6.11	9.31	9.90	15.3	11.10	17.8	10.8
	2.35	4.47	6.43	7.53	8.14	11.4	7.22	13.8	12.2
					14.8		6.80	14.3	
					11.5			12.9	
All Labs:									
$\bar{x}$	3.144	7.156	8.778	11.536	13.909	17.5	9.882	17.686	15.444
s	0.49	1.08	1.19	1.45	2.86	2.45	1.17	2.87	2.49
CV, %	15.6	15.1	13.6	12.6	13.4	14.0	11.8	16.2	16.4
Rec., %	92.7	86.4	85.8	87.6	86.5	87.6			
Acceptable values; data from Laboratories 7 and 9 not used.									
$\bar{x}$	3.247	7.344	9.131	11.957	14.192	17.707	9.996	18.164	15.321
s	0.43	0.81	0.87	0.87	1.44	1.44	0.80	2.68	2.24
CV, %	13.2	11.0	9.5	7.3	10.1	8.1	8.0	14.8	14.6
Rec., %	95.9	88.7	89.3	90.8	88.2	88.6			

[a] Lost sample.

Table 2. Tabulation of linear regression parameters that may be used to establish performance standards

Lab.	Regression eq.	Correlation coeff.	Approx. rec., %
1	$y = 1.0785x - 3.543$	0.9857	93.0
2	$y = 1.1022x - 3.004$	0.9879	90.7
3	$y = 1.0743x - 3.112$	0.9959	93.1
4	$y = 1.1174x - 3.397$	0.9978	89.5
5	$y = 1.0194x - 3.484$	0.9935	98.1
6	$y = 1.2458x - 3.199$	0.9825	80.3
7	$y = 0.9043x - 1.331$	0.9504	110.6
8	$y = 1.2510x - 3.957$	0.9730	79.9
9	$y = 1.2068x - 0.8612$	0.9143	82.9

Table 3. Transformed data from Table 1

Lab.	Sample 1	2	3	4	5	6	7	8	9	Statistic
1	1.298	0.824	1.100	1.110	1.007	1.080	0.980	0.984	0.872	$\bar{x}$ = 1.055
	1.247	1.153	1.102	1.110	1.014	1.040	1.062	1.018	0.931	s = 0.1132
					1.143		1.133	0.984		CV = 10.7
					1.150			0.916		
2	0.954	1.034	0.980	0.902	1.078	1.028	0.998	1.199	0.667	$\bar{x}$ = 0.988
	0.989	1.062	1.082	1.049	0.956	1.074	0.860	1.018	1.096	s = 0.1046
					0.920		0.931	0.973		CV = 10.6
					0.992			0.893		
3	0.849	1.119	1.111	1.066	1.093	1.069	0.891	1.227	1.076	$\bar{x}$ = 1.059
	0.852	1.070	1.038	1.040	1.064	1.034	0.966	1.221	1.050	s = 0.1088
					1.014		1.073	1.300		CV = 10.3
					0.985			1.142		
4	1.056	1.024	1.010	1.049	1.007	1.017	1.083	1.018	1.142	$\bar{x}$ = 1.037
	0.910	1.030	1.065	1.023	1.007	1.017	1.073	1.023	1.030	s = 0.0443
					1.035		1.062	1.001		CV = 4.3
					1.093			1.080		
5	1.043	1.170	1.196	1.075	1.086	1.108	1.083	1.170	1.202	$\bar{x}$ = 1.146
	1.193	1.097	1.139	1.162	1.122	1.097	1.052	1.250	1.096	s = 0.0641
					1.258		1.133	1.261		CV = 5.6
					1.158			1.199		
6	1.018	0.782	0.877	0.954	0.877	0.840	1.052	0.893	1.024	$\bar{x}$ = 0.910
	0.954	0.992	0.854	0.954	1.007	0.960	0.982	0.831	0.832	s = 0.079
					0.834		0.880	0.820		CV = 8.7
					0.899			0.808		
7	1.050	1.070	1.014	0.919	1.100	0.983	1.062	0.746	1.182	$\bar{x}$ = 1.063
	—[a]	1.147	0.994	1.110	0.935	1.326	—[a]	1.057	1.136	s = 0.125
					1.136		1.154	0.905		CV = 11.8
					1.071			1.210		
8	1.104	0.931	1.071	0.936	0.812	0.926	0.994	0.893	1.189	$\bar{x}$ = 0.970
	0.992	1.073	0.933	1.084	1.028	0.874	0.912	0.876	0.957	s = 0.0950
					0.884		1.042	0.865		CV = 9.8
					1.050			0.893		
9	0.744	0.791	0.696	0.807	0.712	0.874	1.123	1.006	0.713	$\bar{x}$ = 0.778
	0.747	0.625	0.732	0.653	0.584	0.651	0.731	0.780	0.806	s = 0.133
					1.064		0.688	0.808		CV = 17.1
					0.827			0.729		
All data:										
$\bar{x}$	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
s	0.157	0.151	0.136	0.126	0.134	0.140	0.1179	0.162	0.164	
CV, %	15.7	15.1	13.6	12.6	13.4	14.0	11.8	16.2	16.4	
Outliers excluded:										
$\bar{x}$	1.033	1.026	1.040	1.037	1.020	1.012	1.012	1.027	1.012	
s	0.137	0.1132	0.0989	0.0752	0.1033	0.0824	0.0813	0.1518	0.1481	
CV, %	13.3	11.0	9.5	7.3	10.1	8.1	8.0	14.8	14.6	

[a] Lost sample.

Table 4. Transformed data with performance outliers excluded

Lab.	Sample 1	2	3	4	5	6	7	8	9	Statistic
1	1.257	0.803	1.058	1.070	0.987	1.067	0.968	0.958	0.861	$\bar{x}$ = 1.030
	1.207	1.123	1.059	1.070	0.994	1.028	1.050	0.991	0.920	s = 0.1074
					1.120		1.120	0.958		CV = 10.4
					1.128			0.892		
2	0.924	1.008	0.942	0.870	1.057	1.016	0.986	1.167	0.659	$\bar{x}$ = 0.966
	0.958	1.035	1.040	1.012	0.937	1.062	0.850	0.991	1.084	s = 0.1012
					0.902		0.920	0.947		CV = 10.5
					0.972			0.870		
3	0.822	1.091	1.068	1.029	1.071	1.056	0.880	1.195	1.064	$\bar{x}$ = 1.034
	0.825	1.043	0.998	1.004	1.043	1.022	0.955	1.189	1.038	s = 0.1056
					0.994		1.060	1.266		CV = 10.2
					0.965			1.112		
4	1.022	0.998	0.972	1.012	0.987	1.005	1.070	0.991	1.129	$\bar{x}$ = 1.014
	0.881	1.004	1.027	0.987	0.987	1.005	1.060	0.996	1.108	s = 0.0474
					1.015		1.050	0.974		CV = 4.7
					1.071			1.052		
5	1.010	1.140	1.150	1.037	1.064	1.096	1.070	1.140	1.188	$\bar{x}$ = 1.119
	1.155	1.069	1.095	1.121	1.099	1.084	1.040	1.217	1.084	s = 0.0609
					1.233		1.120	1.228		CV = 5.4
					1.135			1.167		
6	0.986	0.762	0.843	0.920	0.860	0.830	1.040	0.870	1.012	$\bar{x}$ = 0.889
	0.924	0.967	0.821	0.920	0.987	0.949	0.970	0.809	0.822	s = 0.0795
					0.817		0.870	0.798		CV = 8.9
					0.881			0.787		
8	1.069	0.908	1.030	0.903	0.796	0.915	0.982	0.870	1.175	$\bar{x}$ = 0.948
	0.961	1.046	0.897	1.045	1.008	0.864	0.901	0.853	0.946	s = 0.0926
					0.867		1.030	0.942		CV = 9.8
					1.029			0.870		
$\bar{x}$	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
s	0.133	0.1103	0.951	0.0725	0.101	0.815	0.804	0.148	0.146	
CV, %	13.3	11.0	9.5	7.2	10.1	8.2	8.0	14.8	14.6	

Table 5. Pooled coefficients for variation for NPYR (%)

Labs.	Samples	CV_x	CV_0	CV_b
9	9	14.7	9.5	8.0
9	11	14.8	9.3	8.1
7	9	11.2	8.0	5.5
7	11	11.8	7.6	6.4

sible performance outlier determined for this collaborative study.

The mean, standard deviation, and CV values were redetermined for the data of Tables 1 and 3. For information purposes and to demonstrate that significant differences do not occur, the data of the acceptable laboratories were normalized using the mean of those laboratories. The transformed data are tabulated in Table 4.

The pooled data were analyzed using the method of Steiner (4) to determine the coefficients of variation for repeatability (CV_0), reproducibility (CV_x), and bias (CV_b) and are tabulated in Table 5. Where quadruplicate analyses were done, an additional analysis was done identifying the second set as additional samples. The formula $s_d^2 = 2s_b^2 + s_r^2$ given by Youden (4) was used to determine bias.

The performance standards for the method and the analyst, determined from this study are as follows:

Applicable range of analysis	3–18 ppb NPYR
CV_0 (repeatability)	8%
CV_x (reproducibility)	12%
CV_b (bias)	6%
CV_a (analysts)	11% (n = 23)
r	>0.97 (n = 14)
Av. recovery	80–100% (n = 14)

REFERENCES

(1) ACS Committee on Environmental Improvement (1980) "Guidelines for Data Acquisition and Data Quality Evaluation in Environmental Chemistry," *Anal. Chem.* **52**, 2242–2249

(2) Horwitz, W., Kamps, L. R., & Boyer, K. W. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1344–1354

(3) Greenfield, E. L., Smith, W., Malanoski, A. J. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1316–1318

(4) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA

Comparison of Three Methods for Determination of Crude Protein in Meat: Collaborative Study

FRANCIS B. SUHRE, PAUL A. CORRAO, ANGELINE GLOVER, and ANTHONY J. MALANOSKI
U.S. Department of Agriculture, Food Safety and Inspection Service, Beltsville, MD 20705

Collaborators: T. Dummett; R. Funk; A. Glover; G. Heavner; R. L. Hoover; M. Latham; F. L. Long; J. H. Martini; K. McGee, W. C. Morris; W. Oberste; M. Okamoto; B. Pakrasi; P. J. Pasquarella; J. Reiser; L. Sorensen; J. Lovestrand; M. Taylor; B. Trombella; S. R. Warden; C. Wayo; R. Wiebke; W. Woods

A study was designed to compare the Kjel-Foss automated macro-Kjeldahl method and a block digestion-steam distillation method. The official AOAC Kjeldahl method was used as a reference procedure. Six products with a crude protein range of 10-30% were analyzed by 23 laboratories. Five laboratories analyzed the samples by the official AOAC method, 8 laboratories used the automated Kjel-Foss method, and 11 laboratories used the block digestion with steam distillation method. Standard deviations for each product and each method for both repeatability and reproducibility are given. The block digestion-steam distillation method has been adopted official first action.

Collaborative studies of the block digestor and the automated Kjel-Foss methods have been reported previously (1, 2). The repeatability and reproducibility for meat and meat products, however, must be determined independently because of problems encountered with sample homogeneity. The crude protein official procedure was included as a reference procedure.

Collaborative Study

Six samples were sent to all collaborators as blind duplicates: (*1*) frankfurter, (*2*) pork sausage, (3) ham, (*4*) ground beef, (*5*) lean ground beef, and (*6*) dried beef. Each laboratory was requested to analyze the samples in duplicate on separate days by 3 methods: official final action method **24.027** (3), automated method **24.037** (3), and block digestion-steam distillation method.

This report of the Associate Referee, F. B. Suhre, was presented at the 93rd Annual Meeting of the AOAC, Oct. 15-18, 1979, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee C and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* 64, 427 (1981).

Received April 5, 1982. Accepted June 17, 1982.

Crude Protein

Block Digestion Method – Official First Action

24.B01 ***Reagents***

(**a**) *Catalyst tablets.*—Contg 3.5 g K_2SO_4 and 0.175 g HgO (Kjeltabs "MT" available from Tecator, 1898 S Flatiron Ct, Boulder, CO 80301,or equiv.).

(**b**) *Boric acid soln.*—4%. Dissolve 4 g H_3BO_3 in H_2O contg 0.7 mL 0.1% alc. soln of Me red and 1.0 mL 0.1% alc. soln of bromocresol green, and dil. to 100 mL with H_2O.

(**c**) *Sodium hydroxide-sodium thiosulfate soln.*—Dissolve 2000 g NaOH and 125 g $Na_2S_2O_3$ in H_2O and dil. to 5 L (ca 50 mL is used per analysis).

(**d**) *Hydrochloric acid std soln.*—0.2N (**50.011-50.017**).

24.B02 ***Apparatus***

(**a**) *Digestion block and associated glassware.*—Tecator DS-6 or DS-20 (Tecator), or equiv.

(**b**) *Distillation unit and associated glassware.*—Kjeltec 1003 (Tecator), or equiv.

24.B03 ***Determination***

Accurately weigh ca 2 g well ground and mixed sample on 7 cm N-free filter paper (e.g., Whatman 541), fold, and transfer to 250 mL digestion tube. Place tubes in fume hood and add 2 or 3 boiling stones, 2 catalyst tablets, 15 mL H_2SO_4, and *slowly* 3 mL 30–35% H_2O_2. Let reaction subside and place tubes in block digestor preheated at 410°. (Digestor must be placed in perchloric acid fume hood or be equipped with exhaust system.) Digest at 410° until mixt. is clear, ca 45 min. Remove tubes and let cool ca 10 min. Do not let ppt form; if ppt forms, reheat. Carefully add 50–75 mL H_2O.

Place NaOH-$Na_2S_2O_3$ soln in alkali tank of steam distn unit. Make sure that 50–75 mL is

0004-5756/82/6506-1339-07$01.00

Table 1. Collaborative results for determination of protein by block digestion–steam distillation method (%)

Lab.	Pork sausage		Frankfurter		Ham		Ground beef		Lean ground beef		Dried beef	
1	10.35	11.41	11.22	11.05	16.83	16.98	17.48	17.30	20.91	20.97	30.39	30.95
	10.72	11.48	10.89	11.24	16.45	17.40	17.00	17.29	21.22	21.80	30.71	31.28
	10.31	10.98	11.18	11.06	16.64	17.41	17.08	17.19	20.92	20.92	30.67	30.80
	10.89	11.31	11.13	11.17	16.98	17.19	17.26	16.96	20.75	21.25	30.96	31.03
2	10.53	10.87	10.97	10.99	16.62	17.09	17.24	17.21	20.03	21.18	30.42	30.49
	10.73	10.65	10.96	11.15	17.09	17.14	17.39	17.08	20.73	21.77	30.69	30.05
	10.90	10.75	10.94	10.90	—	16.83	17.55	17.15	20.68	21.47	31.01	30.23
	10.66	10.46	10.78	11.08	—	16.59	17.12	17.12	21.32	21.59	30.76	—
3	10.52	10.72	11.47	11.41	16.62	16.10	17.28	17.73	22.16	21.03	30.64	29.48
	10.76	10.69	10.81	10.94	16.36	16.23	17.01	17.03	19.11	21.32	30.06	29.26
	10.29	10.56	11.24	11.13	15.87	15.88	17.24	17.61	22.10	21.22	30.37	30.77
	10.44	10.35	10.75	11.05	16.59	16.73	17.10	16.81	19.27	21.38	29.49	30.06
4	9.75	10.81	11.06	11.00	16.75	15.94	16.44	17.06	21.06	20.94	30.88	30.06
	10.38	10.75	10.75	11.19	17.00	16.06	17.00	16.69	21.13	21.19	30.00	30.38
	10.50	10.81	11.00	10.87	15.88	16.31	17.13	17.50	20.94	20.50	30.19	30.13
	9.75	10.63	11.00	11.44	17.13	16.31	17.19	17.06	21.19	20.81	30.44	30.13
5	10.41	10.56	10.94	10.94	17.25	16.25	17.30	17.31	20.21	20.81	30.62	30.25
	10.50	10.81	10.77	11.38	16.59	16.75	17.56	17.31	21.94	21.12	30.44	30.56
	10.68	10.69	11.21	11.00	17.38	16.69	17.74	17.00	20.46	—	30.81	29.94
	10.77	10.44	11.12	11.19	16.33	17.00	17.56	17.38	21.81	—	30.38	30.19
6	10.41	10.62	11.09	10.91	16.89	16.50	17.31	17.22	20.94	21.33	30.34	30.29
	10.40	10.54	11.15	11.27	16.33	16.55	17.48	16.97	21.77	20.67	30.44	30.01
	10.34	10.46	11.11	10.95	17.19	16.53	17.40	17.33	20.99	21.26	30.57	30.42
	10.53	10.35	11.06	11.09	16.62	16.59	17.62	17.08	21.79	20.96	30.50	30.05
7	10.69	10.00	11.07	11.42	16.32	15.87	17.57	16.45	20.97	21.35	30.57	29.39
	10.63	10.44	11.62	10.77	17.42	16.89	16.88	17.91	20.75	20.75	30.76	30.48
	10.45	10.38	10.97	10.96	15.87	16.21	17.28	17.63	20.87	21.89	30.28	29.22
	10.40	10.92	11.53	—	17.06	16.35	17.17	17.91	20.81	20.94	30.52	30.55
8	10.52	10.41	11.13	11.20	16.64	16.23	17.11	16.76	21.05	20.73	30.23	30.12
	10.74	10.83	11.32	11.15	15.86	16.08	16.64	17.07	20.71	20.10	29.28	29.72
	10.44	10.98	11.06	11.13	16.37	16.96	16.87	17.32	21.30	20.36	30.51	30.24
	10.92	10.57	11.40	11.32	15.75	16.23	16.63	17.25	21.19	20.48	29.43	29.66
9[a]	10.08	10.02	10.47	10.51	15.20	15.48	15.24	15.95	20.74	20.93	29.55	29.00
	10.42	9.80	10.64	10.38	15.84	15.80	16.01	15.90	20.98	19.48	29.00	29.30
	10.34	10.81	10.60	10.64	15.73	15.84	15.56	16.54	21.04	20.42	29.47	28.85
	10.40	9.55	10.74	10.27	16.35	16.29	15.97	16.31	21.32	20.14	28.53	28.98
10	9.75	10.67	10.70	10.82	15.73	16.47	17.05	17.58	21.30	21.22	30.04	30.81
	10.90	10.41	10.65	11.19	17.53	16.45	18.55	17.37	20.70	20.80	30.92	29.49
	10.80	10.52	11.05	11.35	15.78	16.06	17.70	16.68	20.58	21.04	30.04	30.72
	10.00	10.84	—	11.02	—	16.01	17.15	17.20	—	21.44	30.31	29.84
11	9.09	10.28	10.78	10.75	15.81	15.72	16.44	16.77	19.21	21.08	29.80	30.72
	10.78	10.07	11.03	10.66	16.43	16.26	16.81	17.14	20.87	21.56	29.68	30.76
	10.78	10.41	10.47	10.96	15.97	16.12	16.50	17.35	21.46	20.60	29.50	30.35
	10.47	10.64	11.03	10.66	16.31	16.41	16.70	17.04	—	21.08	29.88	30.90

[a] Outlier laboratory according to Youden (4) and ASTM STP 335 (5).

Table 2. Collaborative results for determination of protein by Kjel–Foss method (%)

Lab.	Pork sausage		Frankfurter		Ham		Ground beef		Lean ground beef		Dried beef	
12	10.67	10.49	11.43	10.35	16.65	16.45	16.96	17.00	20.09	20.42	28.60	28.23
	10.30	10.51	11.44	11.56	14.89	16.25	16.70	17.17	20.37	20.61	28.92	29.14
	10.80	10.21	11.21	10.95	15.71	17.17	17.48	16.28	19.66	20.43	28.46	28.18
	10.61	10.43	10.79	10.29	15.85	15.85	17.02	16.98	20.33	20.42	29.28	29.30
13	10.10	10.38	10.96	11.11	16.48	16.40	17.02	17.33	21.01	21.76	30.00	29.82
	10.42	10.53	11.28	11.12	16.45	16.38	16.53	16.96	20.41	21.01	28.74	29.46
	10.60	10.71	10.99	10.97	16.27	16.74	17.05	17.04	20.90	21.84	29.78	30.00
	10.82	11.00	11.12	11.13	17.03	17.11	16.67	16.71	20.66	20.98	28.64	30.11
14	11.29	10.73	10.65	10.72	16.73	16.20	17.30	17.23	20.62	21.09	29.79	30.61
	10.99	10.56	10.76	11.12	17.07	16.24	17.44	16.94	20.64	20.30	30.91	30.15
	11.33	10.60	10.83	11.08	16.33	16.38	17.83	17.44	20.11	21.45	29.94	30.53
	10.78	10.51	10.55	11.11	16.63	16.71	17.72	17.19	20.95	19.99	30.58	30.36
15[a,b]	10.03	10.57	10.61	10.70	17.18	15.93	16.46	16.68	20.81	20.07	28.66	28.71
	10.01	10.03	10.78	10.64	16.63	16.36	16.39	16.64	21.74	20.18	29.37	29.67
	10.47	10.19	10.63	10.47	17.24	15.84	17.33	16.45	20.23	20.10	29.12	29.84
	10.12	9.79	10.97	10.59	16.18	15.64	16.16	16.60	21.41	19.15	28.92	30.21
16	10.60	10.50	10.60	10.80	17.00	16.10	16.50	17.40	20.40	20.80	29.70	30.10
	10.50	10.80	11.10	11.00	16.00	16.40	16.20	18.0	19.90	19.80	29.70	29.60
	10.30	10.80	10.50	11.00	17.20	16.80	16.80	17.20	20.30	19.90	30.10	29.80
	10.90	10.90	11.00	11.00	15.90	16.10	16.90	17.20	20.20	20.90	29.60	29.80
17	10.75	10.63	11.38	10.81	17.81	16.44	17.19	17.31	21.19	21.19	30.25	30.56
	10.69	10.50	11.50	11.00	16.69	16.69	17.63	17.63	20.69	20.94	30.13	30.75
	10.88	10.94	11.44	10.94	18.19	16.81	16.69	17.25	21.19	21.25	30.25	30.81
	10.56	10.44	11.19	11.00	17.25	16.63	17.44	17.50	21.00	21.13	30.25	30.56
18	10.37	10.50	11.07	10.71	16.56	16.13	17.29	16.85	21.25	21.56	29.78	29.33
	10.31	10.57	11.13	10.85	16.12	16.00	17.08	16.66	21.20	21.69	29.18	30.64
	10.42	10.24	11.03	10.66	16.60	16.49	17.22	16.60	21.30	21.04	29.67	29.71
	10.19	10.22	11.22	10.98	16.50	16.06	16.84	16.61	21.58	21.79	29.18	30.41
19[a]	10.95	10.64	11.33	11.30	17.21	16.98	17.71	17.69	21.10	21.72	30.55	30.15
	11.23	10.88	10.96	11.51	16.36	17.09	16.95	17.35	21.06	23.44	30.99	30.42
	10.90	10.80	11.44	11.25	16.95	16.64	17.52	17.86	21.31	21.13	30.44	30.22
	10.73	11.06	11.35	11.70	16.40	16.80	16.98	17.43	21.92	22.98	30.61	30.11

[a] Outlier laboratory according to Youden (4).
[b] Outlier laboratory according to ASTM STP 355 (5).

Table 3. Collaborative results for determination of protein by official Kjeldahl method (%)

Lab.	Pork sausage		Frankfurter		Ham		Ground beef		Lean ground beef		Dried beef	
20	10.60	10.60	11.20	11.00	15.80	16.30	17.60	17.40	20.90	20.30	30.70	30.60
	10.40	10.40	11.20	10.90	15.90	15.50	17.20	17.50	20.70	20.90	29.70	31.00
	11.10	10.80	11.30	11.10	15.90	16.50	17.20	17.10	21.10	20.40	30.60	30.80
	10.60	10.50	11.30	11.10	15.90	15.80	17.30	17.10	20.40	20.50	29.50	31.10
21	10.79	10.89	11.35	10.89	15.14	15.72	17.83	17.62	21.30	20.37	30.51	29.82
	10.80	10.78	11.44	11.09	15.20	15.16	17.90	17.12	20.77	21.08	30.67	29.76
	10.72	10.87	11.40	10.87	15.16	15.67	17.82	17.64	21.38	20.35	30.50	29.79
	10.73	10.70	11.41	11.13	15.28	15.17	17.90	17.14	20.84	21.14	30.54	29.76
22	10.70	10.70	11.10	11.10	16.10	15.30	17.20	17.20	20.70	20.50	29.80	29.80
	10.80	11.00	11.20	11.10	16.40	16.10	17.70	17.50	22.00	20.90	30.60	30.00
	10.60	10.40	11.10	11.20	16.30	15.40	17.20	17.40	20.60	20.50	29.70	29.30
	10.50	10.80	11.30	11.00	16.40	16.10	17.90	17.50	21.90	20.50	30.70	30.20
23	11.10	10.80	11.40	11.00	15.90	16.10	17.60	17.20	20.60	21.30	30.30	30.40
	10.90	10.40	11.30	11.10	15.90	16.20	17.30	16.90	21.80	21.30	29.80	30.40
	10.70	10.50	11.50	11.10	15.40	16.00	17.60	17.70	21.10	21.70	30.60	30.70
	10.70	10.50	11.60	11.30	16.60	16.20	18.00	17.20	22.10	20.90	29.60	30.60
24	10.49	10.59	10.54	10.94	16.57	16.72	17.04	17.35	20.94	20.90	31.00	30.30
	10.64	10.50	11.03	10.88	16.89	16.14	17.02	17.36	20.47	21.02	29.87	30.70
	10.89	10.76	10.90	11.20	16.57	16.84	17.20	17.34	21.72	21.23	30.91	30.55
	10.99	10.76	11.24	11.14	16.87	16.40	16.73	17.52	20.44	20.84	30.06	30.84

dispensed from unit before conducting distn. Attach digestion tube contg dild digest to distn unit. Place 250 mL receiving flask contg 25 mL H_3BO_3 soln with mixed indicator on receiving platform, with tube from condenser extending below surface of absorbing soln. Steam distil until 100–125 mL collects (absorbing soln turns green from liberated NH_3). Remove digestion tube and receiving flask from unit.

Titr. absorbing soln with 0.2N HCl to neut. gray end point and record vol. acid required to 0.01 mL. Titr. reagent blank similarly.

$$\% \text{ N} = (V_A - V_B) \times 1.4007 \times N/\text{g sample};$$

$$\% \text{ Protein} = (V_A - V_B) \times 1.4007 \times N \times 6.25/\text{g sample},$$

where V_A and V_B = vol. std acid required for sample and blank, resp.; 1.4007 = milliequiv. wt N × 100 (%); N = normality of std acid; and 6.25 = protein factor for meat products (16% N).

Results and Discussion

Data from the collaborative study are given in Tables 1–3. Of the 23 participating laboratories, 11 analyzed the study samples by the proposed method, 8 used the Kjel-Foss method, and 5 reported results for the official Kjeldahl method. One laboratory analyzed the samples by the proposed method and the official Kjeldahl method.

Data were subjected to Youden's ranking test (4) to detect outliers. Outliers were also identified according to ASTM STP 335 (5). No outlying laboratories were determined for the Kjeldahl method when ranked by either technique. Youden's ranking technique identified Laboratory 9 as an outlier for the proposed method, and 15 and 19 as outliers for the Kjel-Foss method. Only Laboratories 9 and 15 were identified as outliers by STP 335.

The tabulated statistical summary is given in Table 4. The standard deviations for repeatability and reproducibility, with the outliers excluded, are acceptable for each of the products. The need for obtaining a homogeneous sample, however, must be re-emphasized.

Recommendations

It is recommended that the proposed method be adopted official first action, and that the automated method, **24.037,** be adopted official final action.

Table 4. Statistical summary[a]

Statistic	Pork sausage			Frankfurter			Ham		
	A	B	C	A	B	C	A	B	C
Kjeldahl									
$\bar{x}$	10.70			11.15			15.99		
S_0	0.16			0.183			0.346		
CV_0	1.50			1.64			2.17		
S_x	0.195			0.201			0.554		
CV_x	1.82			1.80			3.47		
S_0/S_x	0.82			0.91			0.62		
Kjel-Foss									
$\bar{x}$	10.59	10.60	10.65	10.97	10.97	11.05	16.53	16.51	16.55
S_0	0.225	0.221	0.220	0.257	0.295	0.209	0.479	0.501	0.480
CV_0	2.12	2.08	2.07	2.34	2.69	1.89	2.90	3.03	2.90
S_x	0.317	0.306	0.28	0.320	0.295	0.274	0.539	0.547	0.550
CV_x	2.99	2.89	2.63	2.92	2.69	2.48	3.26	3.31	3.32
S_0/S_x	0.71	0.72	0.79	0.80	1.00	0.76	0.89	0.92	0.87
Block Digestion–Steam Distillation									
$\bar{x}$	10.52	10.56	10.58	11.01	11.05	10.93	16.46	16.51	16.52
S_0	0.337	0.337	0.295	0.200	0.206	0.185	0.403	0.426	0.412
CV_0	3.20	3.19	2.79	1.82	1.86	1.69	2.45	2.58	2.49
S_x	0.375	0.355	0.322	0.271	0.224	0.241	0.519	0.480	0.484
CV_x	3.56	3.36	3.04	2.46	2.03	1.96	3.15	2.91	2.93
S_0/S_x	0.90	0.95	0.97	0.74	0.92	0.86	0.78	0.89	0.85
	Ground beef			Lean ground beef			Dried beef		
	A	B	C	A	B	C	A	B	C
Kjeldahl									
$\bar{x}$	17.40			20.96			30.30		
S_0	0.277			0.489			0.496		
CV_0	1.59			2.33			1.64		
S_x	0.328			0.500			0.496		
CV_x	1.89			2.39			1.64		
S_0/S_x	0.84			0.97			1.00		

Table 4. (cont'd)

	Kjel-Foss								
$\bar{x}$	17.07	17.08	17.15	20.88	20.80	29.80	29.80	29.78	29.95
S_0	0.351	0.370	0.305	0.545	0.405	0.379	0.430	0.457	0.400
CV_0	2.06	2.17	1.78	2.61	1.95	1.82	1.44	1.53	1.34
S_x	0.440	0.399	0.383	0.760	0.588	0.587	0.721	0.728	0.519
CV_x	2.58	2.34	2.23	3.64	2.83	2.82	2.42	2.44	1.73
S_0/S_x	0.80	0.93	0.80	0.72	0.69	0.65	0.70	0.63	0.77
	Block Digestion–Steam Distillation								
$\bar{x}$	17.08	17.19	17.17	20.95	21.00	21.00	30.19	30.30	30.30
S_0	0.338	0.331	0.295	0.582	0.630	0.418	0.416	0.449	0.423
CV_0	1.98	1.93	1.72	2.78	3.00	1.99	1.38	1.48	1.40
S_x	0.527	0.367	0.334	0.682	0.63	0.414	0.589	0.476	0.475
CV_x	3.09	2.13	1.95	3.26	3.00	1.97	1.95	1.57	1.57
S_0/S_x	0.64	0.91	0.88	0.85	1.00	1.01	0.71	0.94	0.89

[a] A = All data; B = outliers excluded (ranking according to Youden); C = outliers excluded (according to ASTM STP 335).

Acknowledgments

The Associate Referee thanks the following for their participation in the study:

T. Dummett, Iowa Testing Laboratory, Inc., Eagle Grove, IA 50523

R. Funk, Gerber Products, PO Box 409, Rochester, NY 14602

A. Glover, FSIS, Beltsville, MD 20705

G. Heavner, FSIS, Washington, DC 20250

R. L. Hoover, Central Soya, Inc., Box 112, Gibson City, IL 60936

M. Latham, Allied Chemical Corp., Box 226, Geismar, LA 70734

F. L. Long, Agricultural Research Service, Auburn, AL 36830

J. H. Martini, Institute of Food Science, State Agricultural Experiment Station, Geneva, NY 14456

K. McGee, Armour Co., Box 1427, Ft. Madison, IA 62627

W. C. Morris, A. E. Staley, 2200 Eldorado St, Decatur, IL 62525

W. Oberste, The Pro-Pak Division, H. J. Baker & Bros., Inc., Ft. Smith, AR 72901

M. Okamoto, FSIS, San Francisco, CA 94126

B. Pakrasi, Syntex Research, 3401 Hillview Ave, Palo Alto, CA 94304

P. J. Pasquarella, Hygrade Food Products Corp., 8400 Executive Ave, Philadelphia, PA 19153

J. Reiser, Minnesota Valley Testing Laboratory, Inc., 1411 12th Ave S, Bismarck, ND 58501

L. Sorensen, Moorman Mfg. Co. of California, Inc., Box 1000, San Gabriel, CA 91778

J. Lovestrand, General Mills, Inc., 9000 Plymouth Ave, N, Minneapolis, MN 55427

M. Taylor, FSIS, St. Louis, MO 63102

B. Trombello, E&J Gallo Winery, Modesto, CA 95353

S. R. Warden, Bio Search Labs of San Antonio, Inc., 5430 Fredericksburg Rd, San Antonio, TX 78229

C. Wayo, Gerber Products Co., Fremont, MI 49412

R. Wiebke, Peter Eckrich & Sons, Inc., 1025 Osage St, Ft. Wayne, IN 46801

W. Woods, FSIS, Athens, GA 30604

We also acknowledge the assistance of Michael O'Donnell, Division of Mathematics, Food and Drug Administration, with the statistical evaluation.

References

(1) Noel, R. J., & Hambleton, L. G. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 134–140

(2) Noel, R. J. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 200-291
(3) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA
(4) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA
(5) *ASTM Manual for Conducting an Interlaboratory Study of a Test Method*, ASTM STP 335 (March 1963), American Society for Testing and Materials, Washington, DC

Dual Column Chromatographic Method for Determination of *N*-Nitrosothiazolidine in Fried Bacon

JOHN W. PENSABENE and WALTER FIDDLER
Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118

A dual column chromatographic procedure is described that permits the determination of *N*-nitrosothiazolidine (NTHZ), a newly identified nitrosamine, and *N*-nitrosopyrrolidine (NPYR), the principal volatile nitrosamine, in the same fried bacon sample. The procedure used an acid-Celite, Celite, sodium sulfate column followed by a partially deactivated basic alumina column to isolate NTHZ from bacon for subsequent quantitation by gas chromatography with a thermal energy analyzer (GC-TEA). A limited intralaboratory study gave standard deviations for repeatability and reproducibility of 1.20 and 1.55 ppb, respectively. The recovery of *N*-nitrosothiomorpholine, the internal standard added at the 10 ppb level, was 93.3 ± 6.03%. The method is also free from artifactual nitrosamine formation.

We recently reported the previously unidentified nitrosamine, *N*-nitrosothiazolidine (NTHZ), in fried bacon (1). The amount of NTHZ in bacon is questionable, however, because a general method for its determination is not available. Initially, NTHZ was isolated using extracts from the mineral oil distillation procedure developed by Fine et al. (2). However, the use of this procedure can result in nitrosamine artifacts, especially when residual sodium nitrite is present in fried bacon before analysis (3). The FDA multidetection procedure used (4) for the isolation of volatile nitrosamines was not suitable because NTHZ is not steam-distillable. Therefore, a direct extraction procedure, one that does not result in artifactual nitrosamine formation, was needed to measure NTHZ. We recently reported a rapid screening procedure for the isolation and detection of *N*-nitrosopyrrolidine (NPYR) in fried cure-pumped bacon, using a column chromatographic-thermal energy analyzer technique, which did not result in apparent artifactual nitrosamine formation (3). This method was modified by an additional column chromatographic step to enable the quantitative determination of NTHZ and NPYR from a single fried bacon sample.

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.

Received January 27, 1982. Accepted March 23, 1982.

METHOD

Use caution when handling nitrosamines because they are potential carcinogens.

Apparatus

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

(b) *Chromatographic column.*—Glass, 350 mm long × 32 mm id with 60 mm long × 6 mm id drip tip.

(c) *Chromaflex chromatographic column.*—14.5 mm id × 250 mm long with Teflon stopcock (Kontes Glass Co., No. K-420530-222). Glass-blow a 19/22 joint to top of column if possible.

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

(e) *Tamping rod.*—Glass, 450 mm long with 12 mm diameter disk.

(f) *Gas chromatograph-thermal energy analyzer (GC-TEA).*—Varian Aerograph gas chromatograph Model 2700, or equivalent, interfaced with thermal energy analyzer Model 502. Conditions: 1.8 m × 3.2 mm stainless steel column packed with 15% Carbowax 20M-TPA on 60–80 mesh Gas-Chrom P; helium carrier gas, 40 mL/min; column temperature programmed from 140 to 190°C at 4°/min; injector, 180°C; TEA furnace, 450°C; TEA vacuum, 1.5 mm; liquid nitrogen-ethanol cold trap.

Reagents

(a) *Celite 545.*—Not acid-washed (Fisher Scientific Co.).

(b) *Dichloromethane (DCM), n-pentane and n-hexane.*—Burdick & Jackson Distilled-in-Glass solvents.

(c) *Phosphoric acid.*—6N. Extract once with an equal volume of DCM before use, to remove impurities.

(d) *Sodium sulfate.*—Anhydrous, granular.

(e) *Alumina.*—Basic (Camag); dry in vacuum oven at 140°C for 6 h, and then deactivate with 1.5% water. Store in tightly closed bottle until needed.

(f) *Alundum.*—A. H. Thomas "Boiling stones," 8–14 mesh.

0004-5756/82/6506-1346-04$01.00

(g) *N-Nitrosothiomorpholine (NTMOR) and N-nitrosoazetidine (NAZET) internal standard.*—Each 0.10 μg/mL DCM.

(h) *N-Nitrosothiazolidine (NTHZ) and NTMOR working standard.*—Each 0.10 μg/mL DCM.

(i) *NPYR and NAZET working standard.*—Each 0.10 μg/mL DCM.

NTHZ, NTMOR, NPYR, and NAZET were synthesized from their corresponding amines and sodium nitrite and purified by fractional vacuum distillation according to the general procedure published previously (5).

Sample Analysis

Weigh 10 g Celite into 250 mL beaker. Add 10 mL 6N H_3PO_4, ca 3 mL at a time, and stir Celite with small glass rod until mixture is fluffy and uniform in texture. Using powder funnel, pour resultant acid-Celite mixture into 32 mm id chromatographic column containing glass wool plug at bottom. Insert tamping rod through Celite and tamp from bottom up to achieve a height of ca 25 mm. Accurately weigh 10.0 ± 0.1 g doubly ground fried bacon (passed twice through a 1/8 in. plate) and quantitatively transfer sample to mortar. Add 1.0 mL NAZET-NTMOR internal standard solution (equivalent to 10 ppb) to bacon sample, using 1.0 mL transfer pipet, and then add 25 g sodium sulfate; mix with pestle ca 30 s. Add 20 g Celite to mortar and grind 15–20 s until Celite is thoroughly mixed with sodium sulfate and bacon. Grind with moderate pressure for additional 2 min. Quantitatively transfer free-flowing dry mixture into chromatographic column, and tamp with tamping rod to achieve a total height of ca 100 mm. Add 30 g sodium sulfate to top of column. Rinse mortar and pestle with 10 mL previously prepared pentane-DCM (95 + 5) solvent mixture, and add rinse to column, immediately followed by 90 mL of the same solvent. Begin collection of eluate in 100 mL graduated cylinder. When level of solvent in column just touches top of sodium sulfate, add 125 mL DCM at one time. After 85 mL eluate (Fraction 1) has been collected, change receivers. Collect remaining eluate (Fraction 2) in 250 mL K-D flask and concentrate to 1.0 mL for NPYR-NAZET analysis as described previously (3).

Add 3.0 g alumina to Chromaflex column containing glass wool plug and 15 mL hexane. Add 2.0 g anhydrous sodium sulfate to top of column, and drain hexane to within 25 mm of sodium sulfate. Transfer the 85 mL of eluate (Fraction 1) with 15 mL DCM to 250 mL K-D flask equipped with a 10 mL concentrator tube.

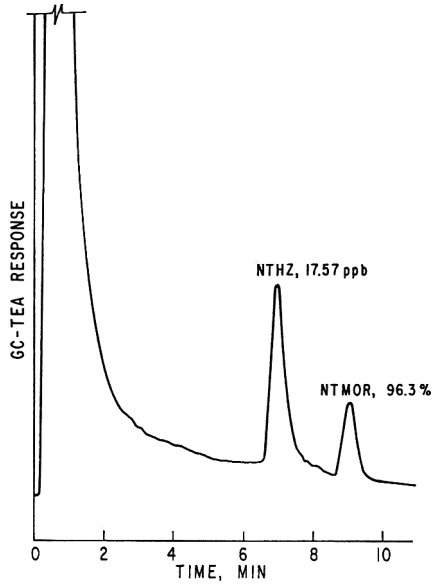


Figure 1. GC-TEA chromatogram from fried bacon extract, dual column method.

Add 2 small boiling stones, attach 3-section Snyder column, and concentrate eluate to 6–8 mL on steam bath (sample generally will not concentrate to less than 6 mL because it contains lipid material from the fried bacon). Dilute sample with 2 mL hexane and quantitatively transfer sample to column, using disposable pipet. Rinse concentrator tube twice with 4 mL hexane and add rinses to column. Collect wash eluate in 125 mL Erlenmeyer flask at rate of 2 mL/min. Add an additional 25 mL hexane to column just as solvent reaches top of sodium sulfate layer. When level of solvent in column again just touches top of sodium sulfate, stop flow and add 125 mL DCM to column. Change receivers, discard first eluate, and collect remaining eluate at flow rate of 2 mL/min in 250 mL K-D flask equipped with 4 mL concentrator tube. Concentrate sample as described previously (3) for determination of NTHZ-NTMOR.

Experimental

The procedure and necessary calculation for determining NPYR and NAZET (Fraction 2) have been described previously (3). NTHZ and NTMOR were determined in a similar manner. Figure 1 shows a typical chromatogram containing NTHZ and the NTMOR internal standard. This method has a minimum detectable level of NTHZ on the GC-TEA system of 1.0 ppb.

Table 1. Statistical analysis for repeatability of *N*-nitrosothiazolidine method

Source of variation	df	Uncorrected NTHZ SS	Uncorrected NTHZ MS	Uncorrected NTHZ F	Corrected NTHZ SS	Corrected NTHZ MS	Corrected NTHZ F	Recovery, % NTMOR SS	Recovery, % NTMOR MS	Recovery, % NTMOR F
N-Nitrosothiazolidine	5	1045.58	209.12	38.72**	1362.70	272.54	150.06**	1058.68	211.74	24.75**
Error	12	64.81	5.40		21.79	1.82		436.77	36.40	
Total	17	1110.50	—		1384.49	—		1495.45	—	
Repeatability[a]			2.32 ppb			1.35 ppb			6.03%	

** $P = <0.01$.
[a] Repeatability = $\sqrt{MS_{error}}$

The procedure used for the mass spectral confirmation of NTHZ in fried bacon extracts was the same as reported previously (1).

Analysis of variance was performed on the measured nitrosamine, according to method described by Snedecor and Cochran (6). Where only the statistical summary is presented, the raw data are available on request. The corrected NTHZ data were adjusted for the recovery of the internal nitrosamine standard.

Results and Discussion

This dual column chromatographic method, hereafter referred to as the "dual column" method, allows for the rapid analysis of the volatile nitrosamines *N*-nitrosodimethylamine and NPYR and the less volatile NTHZ from the same 10 g bacon sample, when the gas chromatograph is temperature programmed. Because NTHZ elutes in the lipid phase, we used a second internal nitrosamine. NTMOR was selected since it had structural and retention characteristics similar to NTHZ, and the possibility of this nitrosamine being naturally present or formed in fried bacon was considered unlikely. In addition, a highly significant correlation ($r^2 = 0.897$, $P < 0.01$) between NTMOR and NTHZ recovery rates was observed in nitrosamine-free fried bacon fortified at the 10 ppb level.

Six samples of fried bacon were analyzed in triplicate to determine the within-laboratory repeatability of this dual column method. Determinations of NTHZ ranged from 9.90 to 34.94 ppb, corrected (7.73 to 34.49 ppb, uncorrected) and recovery of the NTMOR internal standard ranged from 70.9 to 101.3% with a mean of 93.3%. Analysis of variance of the results (Table 1) indicated that the standard deviation of repeatability of NTHZ determination was 1.35 ppb (CV = 6.35%; 2.32 ppb uncorrected) and repeatability of recovery of the NTMOR standard was 6.03% (CV = 6.46%).

NTHZ results for 16 different samples of fried, cure-pumped bacon, which were analyzed by both the dual column and mineral oil methods, are shown in Table 2. The dual column method gave values that ranged from <1 to 26 ppb with an average of 86% lower than the mineral oil method. These results suggest that NTHZ is produced during analysis by the mineral oil method similar to that reported for NPYR (3). To demonstrate that NTHZ is artifactually formed during mineral oil analysis, 3 commercially cured bacon samples containing normally incurred NTHZ were analyzed with the addition of sodium ascorbate and/or α-tocopherol. These compounds are reductants known to destroy nitrite and inhibit nitrosamine formation. The results (Table 3) show that NTHZ formation can be reduced by the addition of inhibitors, but that the NTHZ values are still significantly higher

Table 2. *N*-Nitrosothiazolidine in fried bacon determined by mineral oil and dual column methods

Sample	Nitrosothiazolidine, ppb[a] Mineral oil	Nitrosothiazolidine, ppb[a] Dual column
1	27.91	10.51
2	11.77	4.79
3	26.54	12.24
4	91.40	8.04
5	42.00	14.75
6	184.71	18.93
7	41.92	5.22
8	29.00	8.10
9	65.99	17.41
10	69.70	8.52
11	57.72	7.50
12	26.66	ND[b]
13	282.77	16.93
14	28.04	3.82
15	25.65	11.39[c]
16	197.65	26.20[c]

[a] Corrected values, using NTMOR internal standard.
[b] None detected.
[c] Confirmed by mass spectrometry.

Table 3. *N*-Nitrosothiazolidine inhibition during mineral oil distillation procedure

Inhibitors	Nitrosothiazolidine, ppb[a]		
	Sample 1	Sample 2	Sample 3
None	57.72	26.66	282.77
NaAsc[b]	27.73	21.31	81.65
α-Toc[c]	19.00	14.79	89.41
NaAsc + α-toc	17.68	10.89	44.91
Dual column	7.50	ND[d]	16.93

[a] Corrected for recovery of NTMOR internal standard.
[b] NaAsc: 1500 ppm (aqueous).
[c] α-Toc: 500 ppm (in mineral oil).
[d] None detected.

Table 4. Analysis of variance for intralaboratory study

Variation	df	SS	MS	F
Sample	4	2934.9	733.7	328.30**
Analyst	2	15.8	7.9	3.54
Analyst × sample	8	17.9	2.2	1.54
Error	15	21.7	1.4	
Total	29	2990.3		

** $P<0.01$.

than the dual column method results. Apparently, the rate of nitrosation of the precursor amine to NTHZ is sufficiently great compared with the competitive reaction of nitrite and reductant to form nitric oxide, which is not considered a good nitrosating agent; otherwise, little or no NTHZ would be observed. This indirectly suggests that thiazolidine, a weak base, is the precursor. When these same inhibitors were added to the dual column method, there was no significant change in the NTHZ results, which again indicates that the NTHZ is not being artifactually produced during analysis. Also, the results from a previous experiment (1), where either nitrite and/or cysteamine, a thiazolidine precursor, or thiazolidine itself was added to nitrite-free bacon, show that NTHZ can indeed be artifactually formed as a result of mineral oil analysis.

Finally, a limited intralaboratory study using the dual column method was conducted on fried bacon containing normally incurred NTHZ. The samples were analyzed in duplicate by 3 analysts operating independently. The analysis of variance of the corrected results is shown in Table 4. A significant ($P < 0.01$) difference between the bacon samples was observed with an F-test, as expected, because fried bacon with a wide NTHZ range was observed (2.92–36.60 ppb). No significant analyst effect or analyst × sample interaction was indicated by the analysis of variance. The standard deviations for repeatability and reproducibility were determined as prescribed by Steiner (7) to be 1.20 (CV = 6.64%) and 1.55 ppb (CV = 8.57%), respectively.

The availability of this method will permit the investigation of how NTHZ is formed, so that procedures for its elimination or reduction can be developed.

Acknowledgments

The authors thank Robert Gates and Judith Pascale Foster for their technical assistance, and the National Cancer Institute for the loan of a thermal energy analyzer under contract No. NO1-CP-55715.

References

(1) Kimoto, W. I., Pensabene, J. W., & Fiddler, W. J. (1982) *Agric. Food Chem.* **30**, 757–760.
(2) Fine, D. H., Rounbehler, D. P., & Oettinger, P. E. (1975) *Anal Chim. Acta* **78**, 383–389
(3) Pensabene, J. W., Miller, A. J., Greenfield, E. L., & Fiddler, W. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 151–156
(4) Fazio, T., White, R. H., Dusold, L. R., & Howard, J. W. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 919–921
(5) Pensabene, J. W., Fiddler, W., Dooley, C. J., Doerr, R. C., & Wasserman, A. E. (1972) *J. Agric. Food Chem.* **20**, 274–277
(6) Snedecor, G. W., & Cochran, W. G. (1974) *Statistical Analysis*, 6th Ed., Iowa State University Press, Ames, IA
(7) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA.

DAIRY PRODUCTS

Determination of Chloride Concentration in Cheese: Collaborative Study

L. J. POORTVLIET and WILLIAM HORWITZ[1]
Boter- en Kaascontrolestation "Friesland," Postbus 343, 8901 BC Leeuwarden, The Netherlands

Collaborators: B. M. Krol; E. Garcia Aragonés; J. A. Jans; H. Mair-Waldburg; R. van Renterghem; G. Steiger

Three samples of ground Gouda cheese containing 1-2% chloride were analyzed by 7 laboratories by 3 methods: oxidation with $KMnO_4$ and HNO_3 followed by a Volhard titration; the same but with filtering off the precipitated AgCl before back-titration; and the general potentiometric method without ashing or oxidation. The data were analyzed by ISO statistics (ISO-R 5725) and by AOAC statistics (Youden), the major differences being the rejection of different values as outliers and in the statement of the precision parameters. The within-laboratory variability (repeatability) is comparable for all 3 methods; the between-laboratory variability (reproducibility) is comparable for the Volhard method with filtration and the potentiometric methods, but the direct Volhard method is inferior. Because of its generality and simplicity, the potentiometric method has been adopted official first action; the Volhard method with filtration has been reinstated official final action as an alternative.

For almost 20 years the International Dairy Federation (IDF), the International Organization for Standardization (ISO), and the Association of Official Analytical Chemists (AOAC) have been cooperating in the joint development of methods of analysis for milk and milk products for their own use and for the Committee of Experts on Milk and Milk Products of the FAO/WHO Codex Alimentarius Commission. Fourteen titles in Chapter 16 (Dairy Products) of the 13th edition (1980) of *Official Methods of Analysis* of the AOAC indicate joint endorsement by the 3 organizations.

The current method for the determination of chloride in cheese, **16.242-16.243** (1980), consisting of an oxidation with permanganate and nitric acid, followed by a Volhard titration, was adopted by AOAC after a collaborative study suggested by the joint group (1). This method had served for many years as the Reference Method for ISO (2) and IDF (3). The AOAC method which it replaced, **16.224** (1975), was based on the same principle, but included the additional step of filtration of the precipitated silver chloride before back-titration.

One of the principles of a subsidiary body of the Codex Alimentarius Commission, the Codex Committee on Methods of Analysis and Sampling, is to accept general methods of analysis in preference to specific methods, where possible. Brammell (4) showed that the potentiometric method for the determination of chloride was applicable directly, without ashing or oxidation, to a wide variety of foods with a high degree of precision and simplicity. This method has been recommended as a general method for the determination of chloride in foods to the Codex Alimentarius Commission (5). AOAC has adopted the potentiometric method for the determination of chloride in eggs, **17.027** (1980), canned vegetable products, **32.025-32.030** (1980), and tobacco, **3.137-3.138** (1980). A joint IDF/ISO/AOAC group has now conducted a new collaborative study of methods for the determination of chloride in cheese which included the general potentiometric method as applied to cheese and written in ISO format (6), the current IDF/ISO/AOAC Volhard method, and the same Volhard method but with the silver chloride precipitate filtered off before titration, equivalent to the previous AOAC method.

Chloride in Cheese

Potentiometric Method

Apparatus and Reagents

(a) *Electrodes.*—See **32.026(b)**.

(b) *$AgNO_3$ std soln.*—0.08-0.12N or 0.0856N; see **32.027(b)**.

[1] Bureau of Foods, Food and Drug Administration, Washington, DC 20204.

Received December 30, 1981. Accepted March 26, 1982.
The recommendations presented were approved by the General Referee and Committee C and were adopted by the Association. See *J. Assoc. Off. Anal. Chem.* **66**, March issue (1983).

0004-5756/82/6506-1350-07$01.00

(c) *NaCl std soln.*—0.08–0.12N or 0.0856N; see **32.027**(c).

Standardization

See **32.028.**

Determination

Accurately weigh 2–5 g ground or grated cheese into small blender chamber and add 30 mL H_2O at ca 55°. Suspend cheese by blending; rinse down sides of container with ca 10 mL H_2O. Add 2–3 mL HNO_3 (1 + 3) and titr. with stdzd $AgNO_3$ soln to endpoint; see **32.030.**

Calculation

% Chloride as % NaCl = mL $AgNO_3$ × N $AgNO_3$ × 0.05844 × 100/g sample

% Cl = mL $AgNO_3$ × N $AgNO_3$ × 0.03545 × 100/g sample

Volhard Methods

(a) See **16.242–16.243** (1980). (Method ISO 2970 was furnished for the collaborative study.)

(b) The same method as in (a) but the precipitated AgCl was filtered off before titration.

Collaborative Study

Three samples of ground Gouda cheese with different chloride contents were distributed by the Government Dairy Station, Leiden, The Netherlands, to 9 laboratories. The laboratories were requested to analyze in duplicate by a single analyst, using the 3 methods, Sample 77A1302 on Tuesday, September 6, 1977, Sample 77A1303 on Wednesday, September 7, 1977, and Sample 77A1304 on Thursday, September 8, 1977. Seven laboratories reported results. Laboratory 3 conducted all analyses on the same day, September 9, 1977. In Laboratory 4, 2 analysts, designated 4a and 4b, each conducted all analyses in duplicate.

Results

All reported data are tabulated in Table 1. Outliers as calculated by the AOAC (Youden-Steiner) statistical directions (7) are indicated by underlines; outliers as calculated by the ISO statistical directions (8) are indicated by asterisks. No laboratories were eliminated as being consistently high or low by the AOAC Youden ranking test. Three sets of duplicates, each from a different laboratory but all from Sample 77A1302, were designated as outliers by the AOAC Dixon test as applied to laboratory means.

Table 1. Collaborative results for the determination of chloride (%) in cheese by 3 methods [a]

Lab.	Volhard		Volhard + filtration		Potentiometric	
	Sample 77A1302					
1	0.97	0.99	0.97 *	0.96	1.02	1.02
2	1.02	1.03	1.00	1.00	1.05	1.04
3	1.03	1.01	1.00	0.99	1.02	1.02
4a	0.98	1.01	0.99	1.00	1.00	1.01
4b	1.00	1.00	0.99	0.99	1.00	1.01
5	0.98	0.98	0.98	0.99	0.99	0.99
6	0.87**	0.87	0.99	0.99	1.00	1.00
7	1.02	1.01	1.00	1.00	1.02	1.02
	Sample 77A1303					
1	1.44	1.44	1.47	1.47	1.50	1.50
2	1.49	1.49	1.47	1.47	1.55	1.53
3	1.50	1.52	1.42**	1.46	1.52	1.52
4a	1.48	1.45	1.49	1.49	1.50	1.49
4b	1.46	1.47	1.47	1.48	1.48	1.49
5	1.46	1.48	1.43	1.43	1.47	1.48
6	1.52	1.52	1.53	1.52	1.53	1.53
7	1.47	1.47	1.46	1.47	1.48	1.49
	Sample 77A1304					
1	1.77	1.77	1.81	1.81	1.87	1.87
2	1.83	1.83	1.78	1.79	1.88	1.88
3	1.90	1.88	1.79 *	1.82	1.87	1.87
4a	1.76	1.78	1.82	1.83	1.85	1.83
4b	1.81	1.82	1.84	1.85	1.84	1.83
5	1.81	1.81	1.80	1.80	1.83	1.84
6	1.75	1.75	1.76	1.76	1.86	1.86
7	1.83	1.85	1.82	1.82	1.85	1.85

[a] Outlier indications: *AOAC (Youden-Steiner):* Double column underline is a Dixon laboratory mean outlier; single column underline is a Dixon individual value outlier. *ISO 5725:* ** is an outlier at the 1% critical value by the Dixon test on the mean or the Cochran maximum variance test on the range, and these values are excluded when calculated without outliers; * is a straggler at the 5% critical level and these values are included when calculated without outliers. Laboratory 4a is the same laboratory as 4b but different analysts. In ISO statistics, data from Laboratory 4a were rejected by lot.

Three individual values from 2 of the same sets of duplicates were also designated as outliers by the Dixon test as applied to individual values. The ISO calculations designated 2 of the AOAC sets of duplicates as outliers and in addition indicated an outlier set in each of the other 2 samples.

The statistical parameters for the mean and precision as calculated by the AOAC and ISO statistical directions are given in Table 2. To facilitate comparisons, the precision parameters are expressed both in AOAC terminology (as coefficients of variation (CV)) and in ISO terminology (as prediction intervals). The first line for each sample-method combination gives re-

Table 2. Statistical parameters as calculated by AOAC and ISO procedures [a]

Procedure	N	m	σ_r	σ_R	CV_r	CV_R	r	R	r/R
Sample 77A1302/Volhard									
All values	16	0.986	0.0109	0.0503	1.11	5.11	0.031	0.142	0.22
ISO: omit Labs. 6, 4a	12	1.003	0.0092	0.0209	0.92	2.08	0.026	0.059	0.44
AOAC: omit Lab. 6	14	1.002	0.0116	0.0202	1.16	2.02	0.033	0.057	0.58
Sample 77A1302/Volhard + Filtration									
All values	16	0.990	0.0050	0.0119	0.51	1.20	0.014	0.034	0.41
ISO: omit Lab. 4a	14	0.989	0.0046	0.0124	0.46	1.25	0.013	0.035	0.37
AOAC: omit Lab. 1	14	0.994	0.0046	0.0065	0.47	0.65	0.013	0.018	0.72
Sample 77A1302/Potentiometric									
All values	16	1.013	0.0043	0.0172	0.43	1.69	0.012	0.049	0.25
ISO: omit Lab. 4a	14	1.014	0.0039	0.0180	0.38	1.78	0.011	0.051	0.22
AOAC: omit Lab. 2	14	1.009	0.0038	0.0121	0.37	1.20	0.011	0.034	0.31
Sample 77A1303/Volhard									
All values	16	1.479	0.0106	0.0273	0.72	1.85	0.030	0.077	0.39
ISO: omit Lab. 4a	14	1.481	0.0081	0.0283	0.55	1.91	0.023	0.080	0.29
Sample 77A1303/Volhard + Filtration									
All values	16	1.471	0.0109	0.0302	0.74	2.05	0.031	0.085	0.36
ISO: omit Labs. 3, 4a	12	1.472	0.0049	0.0308	0.33	2.09	0.014	0.087	0.16
Sample 77A1303/Potentiometric									
All values	16	1.504	0.0071	0.0241	0.47	1.60	0.020	0.068	0.29
ISO: omit Lab. 4a	14	1.505	0.0071	0.0258	0.47	1.71	0.020	0.073	0.27
Sample 77A1304/Volhard									
All values	16	1.809	0.0090	0.0460	0.50	2.54	0.026	0.130	0.20
ISO: omit Lab. 4a	14	1.815	0.0081	0.0463	0.45	2.55	0.023	0.131	0.18
Sample 77A1304/Volhard + Filtration									
All values	16	1.806	0.0087	0.0266	0.48	1.47	0.025	0.075	0.33
ISO: omit Lab. 4a	14	1.804	0.0088	0.0276	0.49	1.53	0.025	0.078	0.32
Sample 77A1304/Potentiometric									
All values	16	1.855	0.0061	0.0181	0.33	0.97	0.017	0.051	0.34
ISO: omit Lab. 4a	14	1.857	0.0039	0.0180	0.21	0.97	0.011	0.051	0.22

[a] Symbols: N = number of observations (= number of analysts × 2); m = mean; σ_r = standard deviation (absolute), within-laboratories; σ_R = standard deviation (absolute), between-laboratories (including within-); CV_r = coefficient of variation, within-laboratories; CV_R = coefficient of variation, between-laboratories; $r = 2\sqrt{2} \times \sigma_r$ (repeatability); $R = 2\sqrt{2} \times \sigma_R$ (reproducibility). Units: m, σ_r, σ_R, r, and R are % chloride; N, CV_r, and CV_R are dimensionless.

sults obtained by using all values. Both the AOAC and ISO results are the same. Subsequent lines give the results with outliers removed by the respective directions.

In the case of the ISO procedure, results from Analyst 4a were chosen by lot to be discarded from all sample-method cells, in addition to any mean or range outliers which appeared in the ISO statistical outlier calculations. Therefore, Table 2 always contains a line "ISO: omit [at least] Lab. 4a" for each sample-method combination. In the AOAC statistical calculations, each of the 2 analysts of Laboratory 4 was treated as a separate laboratory, because the results of each analyst were comparable to all of the results and their inclusion added 2 degrees of freedom. We realize that this approach might underestimate the between-laboratory variability but we believe that this effect is not significant.

The data of Tables 1 and 2 are presented vis-

ually in Figure 1. The filled circles are the AOAC outliers indicated in Table 1. The points with the slanted lines are ISO outliers at the 1% confidence level; the ISO outliers at the 5% level (stragglers) are not indicated because they are not excluded in the outlier calculations. The horizontal line for each method-sample combination is the mean calculated from all data for that combination, including outliers. The values for individual data points and means are read on the absolute "% Chloride" scale on the left, repeated for each sample. The CV values are visualized by the bar graphs accompanying each sample-method combination. The first bar (reading from left to right) always represents the CV for all data (no outliers removed). The second bar always represents the CV with ISO outliers removed (Laboratory 4a and any other indicated pairs). The third bar when present (Sample 77A1302 only) represents the CV with AOAC outliers removed. The top of the bar is the composite between-laboratory CV, including within-laboratory CV; the line within the bar is the within-laboratory CV. If the mean is shifted appreciably by the omission of outliers, the corresponding base of the bar is also shifted. All CV values are read on the scale "% CV" near the lower left corner of the figure.

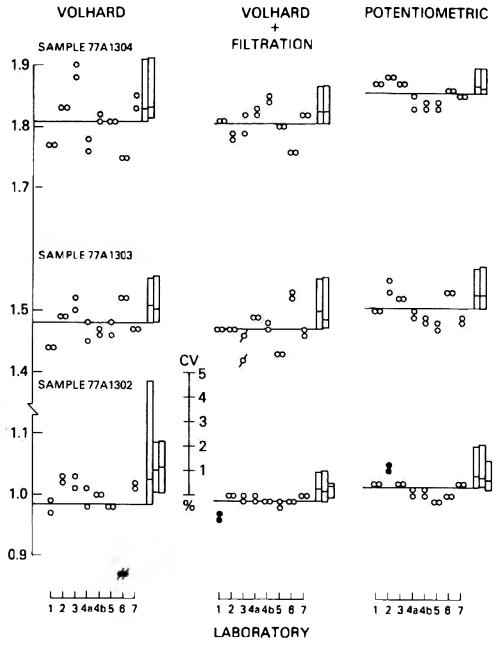


Figure 1. Original collaborative data for percent chloride in cheese from the 3 samples (77A1302, 77A1303, 77A1304) by 3 methods (Volhard, Volhard + filtration, potentiometric) by 7 laboratories (8 analysts). Solid circles indicate outliers by AOAC instructions (Dixon test, 95% confidence level); slant lines indicate outliers by ISO instructions.

Discussion

Both AOAC and ISO treatments give, as they should, the identical mean and standard deviation (SD) when all values are used in the calculations (no outliers deleted). Slight differences in the third (nonsignificant) figure are due to the use of ISO-derived values which were rounded to 2 significant figures. The AOAC statistical procedure either reports precision as an SD as such, or if the precision is a function of concentration, normalizes these values to the dimensionless relative standard deviation (RSD = σ/m, where σ is the appropriate SD and m is the mean) or the coefficient of variation (CV = RSD × 100). The ISO statistical procedure expresses precision as a prediction interval: the value below which the absolute difference between single test results on identical test material may be expected to lie with a specified probability (usually 95%). In other words, assuming a normal distribution of test results, 95% of the absolute differences from the first result of any subsequent test results on the same material will be expected to lie within the calculated interval from the first reading. The precision term involving the same conditions (within-laboratory or within-analyst) is designated as repeatability, r; the precision term involving different conditions (different laboratories, designated as between-laboratories, including within-) is designated as reproducibility, R. The ISO terms are functions of the standard deviation only.

The relationships between the AOAC CV values and the ISO r and R, using duplicates, are:

$$r = 2\sqrt{2} \times \sigma_{within} = 2.83 \times m \times CV_r/100, \text{ and}$$

$$R = 2\sqrt{2} \times \sigma_{between} = 2.83 \times m \times CV_R/100,$$

where the σ values are the within- and between-laboratory standard deviations, respectively, m is the mean, and the CV values are the corresponding coefficients of variation.

As emphasized by Youden, most of the statistical conclusions derived from statistical calculations are apparent by an examination of a plot of the data points themselves, their means (horizontal lines), their scatter, and their CV values (vertical bars), all given in Figure 1. The figure immediately shows the high degree of repeatability of duplicates, the generally greater scatter of the pairs of duplicates about the mean for each

sample-method combination, the substantial displacement of the mean of the potentiometric method, and the radical change in reproducibility with the removal of mean outliers (as distinct from range outliers).

Means.—It is immediately apparent that the mean chloride content by the potentiometric method is significantly higher than the means by the other 2 methods. Because this systematic bias had not been noted in any of the previous collaborative studies comparing the potentiometric method with other methods, no provision had been made in the experimental design to determine the cause of the difference. The largest difference is 0.05% chloride at the 1.8% level.

Outliers.—The major difference between the AOAC statistical treatment and the ISO treatment of the data is the handling of outliers. The AOAC statistical procedure first applies a ranking test for consistently high or low laboratories and then a Dixon test to individual laboratories or values. The Dixon test consists of comparing the difference of the extreme value from its nearest neighbor (sometimes its next-to-nearest neighbor) to the range of the entire set (sometimes the range is reduced by 1 or 2 values). All outlier tests are applied at the 95% probability level.

In ISO statistics, the Dixon test is applied only to the means of the duplicates and then a Cochran maximum variance test is applied to the ranges of duplicates. The Cochran test examines the ratio of the square of the maximum difference between duplicates to the sum of the squares of all of the ranges of the set. With both the Dixon and Cochran tests, if the test statistic lies between the 5% and 1% critical values, the tested value is called a "straggler" and is marked with a single asterisk and the value is included in all calculations. If the test statistic is greater than the 1% critical value, the tested value is called a statistical outlier, marked with a double asterisk, and is omitted from the subsequent calculations.

Thus the 2 statistical procedures examine different things. The AOAC procedure emphasizes consistent and marked deviations and de-emphasizes replication as knowns; the ISO procedure emphasizes marked mean deviations and large differences among replicates and assumes that replication as knowns are independent, an assumption Youden specifically rejects. The difference in viewpoint thus leads to rejection of different values as outliers, but the reasons for rejection are more easily seen in the figure than from the tables.

No laboratory of the present study is a rank outlier. Both statistics reject the pair of values of Laboratory 6, Sample 77A1302, Volhard method. These values are far from the overall mean calculated with these values included. This is the only set where omission of the pair of outliers changes the mean significantly. The pair of duplicates from Laboratory 1, same sample, Volhard method with filtration, is a Dixon laboratory outlier by AOAC statistics and a straggler by ISO statistics. It gains the outlier distinction in this set because the values from the other laboratories are very close together. The value from Laboratory 2, same sample, potentiometric method, is an outlier by AOAC statistics for the same reason. It is not even a straggler by ISO statistics. Laboratory 3, Sample 77A1303, Volhard method with filtration, is an ISO statistical outlier because of the relatively large difference between duplicates, a condition not tested by AOAC statistics. Laboratory 3, Sample 77A1304, Volhard method with filtration, is an ISO straggler for the same reason.

Precision.—The repeatability (within-laboratory variability or differences between duplicates in the individual laboratories) appears to be comparable for all 3 methods and for all 3 samples. The reproducibility (between-laboratory variability or differences among the means of duplicates from the various laboratories) appears to be comparable for the Volhard method with filtration and the potentiometric method. By inspection of the figure, the direct Volhard method is definitely inferior (shows more variability between laboratories) to the other 2 methods.

Other interesting observations which are apparent from Figure 1 and verified in general by reference to the parameters of Table 2 are that there is less variability between-laboratories in Sample 77A1302 than in the other 2 samples and there is less variability in the potentiometric method than in the other 2 methods, and that, in general, the duplicates are very close together.

Comparison with previous study (1).—There are several marked differences in precision characteristics of the same methods between this and the previous study, as shown in Table 3. The repeatability and reproducibility in this study are considerably lower, in several cases by a factor of 2. Similarly, the ratio of the repeatability to reproducibility is quite different: almost 0.6 in the previous study and about 0.3 in the present study. A possible explanation is the use of more experienced analysts and more uniform samples in the present study, although a ratio of 0.5–0.7

Table 3. Statistical parameters of the 2 studies of determination of chloride in cheese

Method	Labs × samples × replicates	m	$\overline{CV_r}$	$\overline{CV_R}$	r/R	% Outliers
		Volhard				
Present study:	8 × 3 × 2 = 48	1.425	0.78	3.17	0.25	0
Omit AOAC outliers	46	1.430	0.79	2.14	0.37	4
Omit ISO outliers	7 × 3 × 2 = 42	1.433	0.64	2.18	0.29	5[a]
Previous study (1)	7 × 5 × 2 = 70	2.201	1.67	2.60	0.64	0
Omit AOAC outliers	6 × 5 × 2 = 60	2.197	1.85	2.73	0.68	14
		Volhard + Filtration				
Present study:	8 × 3 × 2 = 48	1.422	0.58	1.57	0.37	0
Omit AOAC outliers	46	1.424	0.56	1.42	0.39	4
Omit ISO outliers	7 × 3 × 2 = 42	1.422	0.43	1.62	0.27	5[a]
Previous study (1)	7 × 5 × 2 = 70	2.214	1.41	2.98	0.47	0
Omit AOAC outliers	6 × 5 × 2 = 60	2.201	1.49	2.60	0.57	14
		Potentiometric				
Present study:	8 × 3 × 2 = 48	1.457	0.41	1.42	0.29	0
Omit AOAC outliers	46	1.456	0.39	1.26	0.31	4

[a] The omission of Lab. 4a by lot is not considered an ISO statistical outlier. Symbols: $\overline{CV_r}$ = arithmetic average of CV_r of Table 2; $\overline{CV_R}$ = arithmetic average of CV_R of Table 2; other symbols as in Table 2.

is characteristic of most collaborative studies. The previous study also contained 14% outliers because one of the laboratories was a rank outlier, compared with 4–5% outliers, when present, in the present study.

The differences in precision indices between the 2 studies illustrate the fact that these indices too have their own statistical distribution, and a single collaborative study may not provide a representative set of statistical parameters. Nevertheless, it would appear that the reproducibility of the potentiometric method is adequate for its intended purpose of controlling the salt content of cheese. Because the absolute chloride content in the cheese samples is not known, no conclusion can be drawn with regard to bias.

Recommendation

It is recommended that the potentiometric method for the determination of chloride in cheese be adopted official first action as a reference method to replace the present official final action method, **16.242–16.243,** which should be repealed official first action; further that method **16.224** of the 12th edition (1975) be reinstated official final action as an alternative method.

Acknowledgments

The authors gratefully acknowledge the following members and non-members of the Joint IDF/ISO/AOAC group who participated in this study: B. M. Krol, Rijkszuivelstation, Leiden, The Netherlands; E. Garcia Aragonés, Laboratorio Agrario Regional de Andalucia Occidental, Cordoba, Spain; J. A. Jans, Zuivelcontrole-Instituut, Leusden, The Netherlands; H. Mair-Waldburg, Milchwirtschaftliche Untersuchungsund Versuchsanstalt, Kempten, West Germany; R. van Renterghem, Rijkszuivelstation, Melle, Belgium; G. Steiger, Eidgenössische Forschungsanstalt fur Milchwirtschaft, Liebefeld-Bern, Switzerland.

The authors are also very grateful to G. A. Werdmuller, Rijks Kwaliteits Instituut voor Landbouw- en Tuinbouwprodukten, Wageningen, The Netherlands, and Richard Albert, Food and Drug Administration, Washington, DC, for statistical analysis of the data.

References

(1) Brzenk, H. R., & Krett, O. J. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 1142–1145

(2) International Organization for Standardization ISO 2970-1974(E). Available from American National Standards Institute, 1430 Broadway, New York, NY 10018, and other national standards organizations

(3) International Dairy Federation IDF Standard No. 17A: 1972. Available from International Dairy Federation, Square Vergote, Brussels, Belgium

(4) Brammell, W. S. (1974) *J. Assoc. Off. Anal. Chem.* **57**, 1209–1216

(5) Codex Alimentarius Commission, Report of the Thirteenth Session, Rome, 3–14 December 1979, Sections 239–240; Report of the Eleventh Session of the Codex Committee on Methods of Analysis

and Sampling, Budapest, 2–6 July 1979, Appendix IV. Food and Agriculture Organization of the United Nations, Via delle Terme di Caracalla, 00100 Rome, Italy

(6) International Organization for Standardization Draft International Standard ISO/DIS 5943. Subsequently replaced by ISO 5943. Available from American National Standards Institute, 1430 Broadway, New York, NY 10018, and other national standards organizations

(7) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA

(8) International Organization for Standardization International Standard ISO 5725. Available from American National Standards Institute, 1430 Broadway, New York, NY 10018, and other national standards organizations

Evaluation of CH Stretch Measurement for Estimation of Fat in Aqueous Fat Emulsions Using Infrared Spectroscopy

BERNICE L. MILLS and F. R. VAN DE VOORT
University of Guelph, Department of Food Science, Guelph, Ontario, Canada N1G 2W1

Measurement of infrared absorption by the use of a CH stretch filter which isolates wavelengths in the order of 3.4-3.5 μm has been suggested as a replacement for the conventional carbonyl stretch absorption measurement or as an additional measurement for the estimation of fat in aqueous fat emulsions. To determine the merits of this CH stretch measurement, a study was conducted with a series of natural fats selected for their differences in average molecular weight, fatty acid composition, and degree of unsaturation. In this study, the use of the CH stretch filter, the C=O stretch filter, or the combination thereof could predict the fat content with equal accuracy if the fat was consistent in average molecular weight and degree of unsaturation. For a fat which varied in its degree of unsaturation, the CH filter measurement could be used in conjunction with the iodine value to produce accurate results. The combination C=O filter, CH filter, and the iodine value produced the most accurate results when the intent was to analyze a variety of fats on a single calibration.

The use of infrared (IR) spectroscopy in food analysis has been limited in spite of its wide application in other areas of organic chemistry. This can be attributed primarily to the complexities of sample preparation and to the vital role played by water in most food systems. Water shows very strong absorption bands throughout the IR region so that, in spectra of aqueous solutions, absorption bands due to the components are usually obscured unless special instrumentation is used. Recent developments in instrumentation have resolved some of the practical difficulties involved in aqueous solution spectroscopy and IR spectroscopy is now finding increased use in the direct quantitative study and analysis of various food components.

Goulden is credited with the first major contribution to the practical application of quantitative IR spectroscopy to a food system. In 1961, Goulden applied for a United Kingdom patent for the quantitative analysis of milk by infrared absorption based on a study in which 20 milk samples were analyzed in an IR double-beam spectrophotometer (1). The spectra clearly showed absorption peaks at 5.73 μm (1745 cm^{-1}), 6.46 μm (1548 cm^{-1}), and 9.6 μm (1042 cm^{-1}), due to absorption of infrared energy by the carbonyl ester (C=O) linkages of the fat molecules, by the peptide (CONH) linkages of the protein molecules, and by the hydroxyl groups (OH) of the lactose molecules, respectively. A commercial grating instrument, the IRMA (Infrared Milk Analyzer), employing Goulden's principle was developed and the method was officially adopted in 1972 (2). Since then, a number of IR instruments have been developed and marketed, which use filters rather than gratings for selecting wavelengths.

The infrared method has emerged as the technique most suited for wide scale analysis of milk and has had a tremendous impact on the dairy industry. Payment schemes for dairy farmers in many countries are based on IR analysis of milkfat. The success of the IR method in analyzing milk has been due in great part to the relatively consistent composition of cow's milk. However, there has been increasing concern with reports on IR analysis of anomalous milks or milks from cows fed protected polyunsaturated supplements.

The feeding of formaldehyde-protected sunflower-soybean supplements (3) or protected tallow (4) influences the accuracy of fat analysis of milk by the carbonyl ester linkage filter. Deviations as large as −0.3% milkfat have been reported (4). In an attempt to minimize these variations, measurement of absorption by the use of a CH stretch filter which isolates wavelengths in the order of 3.4-3.5 μm has been recommended for fat determination (5). The CH absorption measurement has been suggested either as a replacement for the conventional C=O absorption measurement or as an additional measurement to correct for possible discrepancies at the C=O absorption band.

Theory

The IR absorption by the carbonyl group (C=O) in the ester linkage of triglyceride molecules is proportional to the number of triglyceride molecules and, hence, proportional to the concentration of the fat. This basic principle first presented by Goulden is correct if the mean

Received January 27, 1982. Accepted April 1, 1982.

0004-5756/82/6506-1357-05$01.00

molecular weight of the fatty acids constituting the triglycerides is constant. If, however, the average chain length (mean molecular weight) of the fatty acids is increased, the number of triglyceride molecules per unit weight of fat will decrease and the absorption by the C=O group will not be proportional to the concentration of the fat. One can state, therefore, that the absorption by the carbonyl bond is influenced by variations in the mean molecular weight of the fat. In the case of milk from cows fed protected lipid supplements, the marked increase in linoleic acid (C18:2) is accompanied by an increase in stearic (C18:0) and oleic (C18:1) acids and by a decrease in palmitic (C16:0) and other fatty acids of lower molecular weight (6). This produces a lower IR reading at the 5.73 μm C=O wavelength relative to normal milks unless the instrument has been calibrated specially for these types of milk.

In the 3.3–3.5 μm region, infrared absorption bands arise from CH stretching modes of organic materials, a fact which was recognized as early as 1905 by Coblentz (7). Both CH3 and CH2 groups absorb in this region of the spectrum, the former at 3.38 μm (2962 cm^{-1}) and 3.48 μm (2872 cm^{-1}), and the latter at 3.42 μm (2926 cm^{-1}) and 3.50 μm (2853 cm^{-1}) (7). High resolution quantitative work in this region therefore affords a method for the estimation of the relative numbers of CH3 and CH2 groups in hydrocarbons. However, in low resolution instruments, as in the case of IR milk analyzers, there is an overlapping of absorption bands, which makes it difficult to accurately measure the absorptivities of the individual groups. While the C=O absorption is not materially affected by unsaturation (8), the CH stretching absorption is markedly reduced by the presence of double bonds adjacent to CH2 and CH3 groups (9–11). It has also been noted by Pozefsky and Coggeshall (12), that the absorptivities of CH3 and CH2 groups in the CH stretch region are not the same for oxygen-containing materials, so that the correlation between CH stretch intensity and molecular weight cannot be applied to non-hydrocarbons. This has been confirmed by Francis (13) who found a marked decrease in the integrated absorption intensities of methyl or methylene groups adjacent to an oxygen atom or a carbonyl group.

To determine the merits of the CH stretch filter, a study was conducted with a series of natural fats selected because of their differences in average molecular weight, fatty acid composition, and degree of unsaturation.

Experimental

A Multispec M infrared milk analyzer (Berwind Instrument Group, Birmingham, MI) was interfaced directly to a Hewlett-Packard 9815S desk computer; data was processed by a program package developed by D. A. Biggs (personal communication, 1980). The instrument was fitted with 2 filters, one having a center wavelength of 3.48 μm for the sample beam and another having a center wavelength of 3.56 μm for the reference beam.

Seven natural fats and oils, one of which had been selectively hydrogenated to 3 varying degrees, included the following: refined and bleached coconut, corn, palm, peanut and soybean oils; slightly, moderately, and highly hydrogenated soybean oils; cold pressed olive oil and butter oil. Nitrogen was bubbled through the samples when received, and stored at −20°C to prevent autooxidation during storage. An emulsion of each fat was prepared at ca 6.0% (w/w) concentration, using an emulsifying solution containing 0.6% of a Span 80–Tween 80 mixture (65 + 35). To ensure an average particle size of about 1 μm, a Polytron high-speed mixer (Brinkmann Instruments) was used to prepare the emulsion and the mixture was then homogenized using the Multispec external homogenizer. Nine additional serial dilutions of each emulsion were prepared by mixing appropriate proportions of emulsion and emulsifying solution to give concentrations ranging from about 0.6 to 5.4%.

Two independent series of emulsions were prepared and analyzed on separate days. The samples were placed in a thermostated bath set at 42.5°C before measurement. The instrument was optically zeroed with the stock emulsifier solution, and only the primary uncorrected CH and C=O signals were recorded for each serial dilution of the fats. The use of uncorrected signals obviates any bias introduced by a particular calibration.

Duplicate determinations for actual fat content were done on the 6% emulsions and the emulsifying solution, using the standard reference Mojonnier method (14). The fat content of the emulsion was calculated as the Mojonnier result for the emulsion less the Mojonnier result for the emulsifying agent. Some emulsifying agent was lost to the aqueous phase during the Mojonnier extraction, introducing a small but constant error in the Mojonnier results. Fat content of the serial dilutions was calculated by multiplying the fat content of the 6% emulsion by the appropriate dilution factor.

Table 1. Slope, intercept and standard error of the estimate (SEE) obtained by linear or multiple regression of the chemical results vs the instrumental results

Fat	CO Filter			CH Filter			CO + CH Filters,
	Slope	Intercept	SEE	Slope	Intercept	SEE	SEE
Corn	1.145	−0.048	0.024	1.745	−0.002	0.027	0.023
Peanut	1.137	−0.057	0.022	1.635	−0.013	0.023	0.016
Olive	1.135	−0.039	0.031	1.582	−0.003	0.039	0.032
Soybean	1.134	−0.069	0.014	1.797	−0.017	0.039	0.014
Soybean 1[a]	1.128	−0.065	0.035	1.632	−0.016	0.028	0.027
Soybean 2[a]	1.126	−0.065	0.029	1.550	−0.004	0.030	0.013
Soybean 3[a]	1.116	−0.072	0.027	1.536	−0.030	0.022	0.018
Palm	1.097	−0.071	0.024	1.503	−0.001	0.049	0.025
Butter	0.924	−0.052	0.016	1.489	−0.012	0.023	0.016
Coconut	0.829	−0.038	0.014	1.515	−0.007	0.018	0.013

[a] 1, Slightly hydrogenated; 2, moderately hydrogenated; 3, highly hydrogenated.

Fatty acid composition of the fats was determined by gas-liquid chromatography (GLC) of the methyl esters. The fatty acid methyl esters were prepared by transesterification using sodium methoxide in alcohol. A Hewlett-Packard 7620 gas chromatograph equipped with a 1/8 in. stainless steel column packed with 5% ethylene glycol adipate on 80–100 mesh acid-washed Chromosorb G was used for the analysis. Helium was used as the carrier gas and the column was run isothermally (195°C) with the injection port temperature maintained at 220°C and the flame ionization detector at 250°C. The instrument was calibrated for quantitative analysis by using pure methyl ester standards. The iodine value (IV) and saponification number (SN) were calculated from the GLC data.

Results and Discussion

To increase the scope of inference of the experiment and the degrees of freedom for statistical analyses, the 2 independent sets of results were combined. Linear and multiple regression data are presented in Table 1. The intercepts for linear regression are all negative, indicating a small consistent instrument bias. The standard errors of the estimate (SEE) for the combined use of filters are of the same order of magnitude as the SEE values for the C=O or the CH filters and of the same order of accuracy as those reported by van de Voort (15). This would indicate that the C=O filter, the CH filter, or the combination thereof can be used for the determination of fat content when the instrument is calibrated with the type of fat to be analyzed.

To determine whether a single calibration may be satisfactory for all types of fat, slopes of the individual fats were compared using a Student's t-test with $(n1 + n2 - 4)$ degrees of freedom for homogeneity of regression (16). For the C=O filter, 8 of the 10 slopes were considered to be estimates of a common slope. The slopes for butter and coconut oils were significantly different from the 8 other oils. When linear regression was performed on the group of 8 oils, a standard deviation of the difference (SDD) of 0.060 indicated that, on the single calibration, it was possible to use the C=O filter for several oils and fall within the specifications for accuracy (17). The common denominator of the 8 fat samples is their similarity in molecular weight or saponification numbers, i.e., ca 200. Butter oil and coconut oil have significantly higher saponification numbers and therefore cannot be estimated with this same calibration.

Although the C=O absorption is not materially affected by the presence of double bonds, a gradual change in signal is noted in the case of the 4 soybean oils (Table 2). The change, which is not significant, is probably due to the presence of a small amount of conjugated double bonds. During hydrogenation, migration of double

Table 2. Saponification number (SN), iodine value (IV), and theoretical signals obtained for CH and C=O filters for 5% fat, based on slopes and intercepts presented in Table 1

Fat	SN	IV	CH signal	CO signal
Corn	201.28	132.27	2.87	4.41
Peanut	197.82	101.35	3.07	4.45
Olive	200.80	84.56	3.16	4.44
Soybean	201.42	137.83	2.79	4.47
Soybean 1	200.72	99.09	3.07	4.49
Soybean 2	200.30	80.34	3.23	4.50
Soybean 3	200.40	71.13	3.27	4.54
Palm	206.83	55.41	3.33	4.62
Butter	230.00	30.69	3.37	5.47
Coconut	256.54	9.00	3.30	6.08

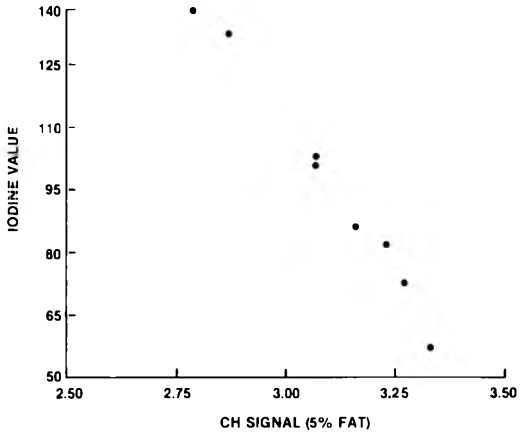


Figure 1. Plot of iodine value vs CH signal for 5% fat, illustrating dependence of CH signal on iodine value of fats of similar average molecular weight.

bonds occurs and there is some conversion of unconjugated double bonds to conjugated double bonds. Conjugation produces a small shift in the C=O frequency (7) and lowering of the frequency could explain the small signal change with hydrogenation of the soybean oils.

When the *t*-test for differences between slopes was performed on the CH filter data, homogeneity of regression was obtained for butter, palm, and coconut oils. The differences in unsaturation between these oils should have resulted in a difference in respective CH slope values and consequently in the CH signal; however, the effect of differences in unsaturation was cancelled by converse differences in mean molecular weight. On a weight basis, the shorter the average chain length of the fat, the greater the proportion of glyceryl and carbonyl groups. The CH2 groups in the glyceryl portion of the triglyceride are adjacent to oxygen and a carbonyl group and contribute little to the CH stretch absorption (7). Hence, coconut oil with a high saponification number and a low iodine value produces a CH signal of the same relative intensity as palm oil with a lower saponification number and a higher iodine value. In the case of the soybean oils, where the mean molecular weights are the same, the CH signal decreases as a function of the degree of unsaturation. This would indicate that an estimate of unsaturation or the iodine value would be required to adjust the slopes to a common value. The direct relation between iodine value and the CH signal can be seen in Figure 1 when the mean molecular weight is kept relatively constant as in the case of the first 8 fats from Table 1. Also, if one performs a linear regression for all the oils, a SDD value of approximately 0.20 is obtained for the CH filter data. However, one can significantly improve this value (0.219 to 0.076) by including the iodine value as a variable in the regression.

The combined C=O and CH filter data for all the fats were analyzed by multiple regression with an overall SDD value of 0.220. When the iodine value was added as another variable along with the combination of filters, a value of 0.027 instead of 0.220 was possible. Of all the combinations investigated, the use of the C=O filter along with the CH filter and iodine value produced the most accurate results.

Conclusion

On a single calibration, the use of the CH stretch filter (3.48 μm), the C=O stretch filter (5.73 μm), or the combination thereof can predict the fat content with equal accuracy if the fat is well defined and consistent in average molecular weight and degree of unsaturation. In this study, the C=O filter was more useful than the CH filter because most of the fats investigated had similar molecular weights and varied in their degree of unsaturation. The C=O absorption is not materially affected by unsaturation and, although affected by chain length, it will tolerate differences in molecular weight of about 10 saponification units before the results become unacceptable. An estimate of mean molecular weight or the saponification number of a fat can be used to group fats or oils for analysis with the C=O filter if only one calibration is to be used.

The absorption at the CH stretch band is significantly reduced by the presence of double bonds and the CH filter is useful in combination with the C=O filter when the increase in chain length does not involve an increase in unsaturation. In the case of anomalous milks, the CH filter would correct for chain length differences, if the increase in chain length does not also correspond to an increase in degree of unsaturation, i.e., an increase in C18:2 and C18:1 fatty acids. For milks or fats which vary in their degree of unsaturation, the CH filter should be used in conjunction with the iodine value to produce accurate results. The combination of C=O and CH filters with the iodine value produces the most accurate results when the intent is to analyze a variety of fats on a single calibration.

For the purpose of this work, fat emulsions were used as models without taking into account interferences from other components which may

be present in real food systems. However, the inferences obtained from this study give some insight into the practical use of instruments equipped with one or both of these filters.

Acknowledgments

The authors thank W. G. Mertens and C. K. Cross of Canada Packers Ltd for their technical assistance and for supplying the fat samples, and the Berwind Instrument Group for supplying the Multispec M infrared milk analyzer for this study. We also acknowledge the valuable advice given by D. A. Biggs and the technical assistance of J. Biggs.

References

(1) Goulden, J. D. S. (1961) *Nature* **191,** 905–906
(2) Biggs, D. A. (1972) *J. Assoc. Off. Anal. Chem.* **55,** 488–497
(3) Dunkley, W. L., Franke, A. A., & Smith, L. M. (1977) *J. Dairy Sci.* **60,** 45–52
(4) Franke, A. A., Dunkley, W. L., & Smith, N. E. (1977) *J. Dairy Sci.* **60,** 1870–1874
(5) Nexo, et al. (1981) US Patent 4,247,773, January 27
(6) MacDonald, I. W., & Scott, T. W. (1977) *World Rev. Nutr. Diet.* 144–154
(7) Bellamy, L. J. (1975) *The Infra-red Spectra of Complex Molecules,* Chapman & Hall, London, UK
(8) Kauffman, F. L. (1964) *J. Am. Oil Chem. Soc.* **41,** 4–42
(9) Sinclair, R. G., McKay, A. F., Myers, G. S., & Jones, R. N. (1952) *J. Am. Oil Chem. Soc.* **74,** 2578–2885
(10) Keeney, P. G. (1962) *J. Am. Oil Chem. Soc.* **39,** 304–305
(11) Arnold, R. G., & Hartung, T. E. (1971) *J. Food Sci.* **36,** 166–168
(12) Pozefsky, A., & Coggeshall, N. D. (1951) *Anal. Chem.* **23,** 1611–1619
(13) Francis, S. A. (1951) *J. Chem. Phys.* **19,** 942–948
(14) Mojonnier Bulletin 101, Mojonnier Bros. Co., Chicago, IL
(15) van de Voort, F. R. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 973–980
(16) Steele, R. G. D., & Torrie, J. H. (1960) *Principles and Procedures of Statistics,* McGraw-Hill Book Co. Inc., New York, NY
(17) Biggs, D. A. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 1211–1214

COSMETICS

High Pressure Liquid Chromatographic Determination of Allantoin in Cosmetic Creams and Lotions

KAZUO NAKAO, KEIICHI HONDA,[1] and TOHRU YONEYA
Kanebo, Ltd, Cosmetics Laboratory, 5-3-28 Kotobuki-cho, Odawara 250, Japan

A sensitive high pressure liquid chromatographic method was developed for the determination of allantoin in cosmetic preparations. The procedure consists of simple cleanup of samples, derivatization with *p*-nitrobenzaldehyde in *N,N*-dimethylformamide to an ultraviolet labeled derivative, and reverse phase chromatography on an octadecylsilylated silica column. Ultraviolet absorbance was measured at 270 nm. Recovery was greater than 97% for cosmetic samples, and the minimum limit of detection was 10 ng.

Allantoin has long been used as a topical remedy for treating suppurating wounds, resistant ulcers, and similar conditions because this compound stimulates the growth of healthy tissues (1). Allantoin is also widely used in cosmetics to promote the renewal of damaged or abraded skin.

Most methods that have been adopted for the quantitative determination of allantoin in plants, serum, or urine (2–4) are essentially based on the Rimini–Schryver reaction presented by Young and Conway (5), which involves the following successive steps: (a) alkaline hydrolysis of allantoin to allantoic acid, (b) acid hydrolysis of allantoic acid to glyoxylic acid, and (c) subsequent estimation of glyoxylic acid by spectrophotometric measurement of a chromophore produced by condensation of glyoxylic acid with phenylhydrazine, followed by oxidation of the hydrazone with ferricyanide. Instead of phenylhydrazine, Borchers (6) used 2,4-dinitrophenylhydrazine to obtain a more stable chromophore. Sumi et al. (7) and Kaito et al. (8) adopted fluorometry, using glyoxylate reductase or phenylephrine hydrochloride in determining glyoxylic acid derived from allantoin in plasma or pharmaceutical preparations. Because the colorimetric methods, including fluorometry, are susceptible to the influence of contaminating chromogens, Christman et al. (9) pointed out the desirability of separating allantoin from manifold contaminants before analysis. All of these procedures are time-consuming, and must be performed with care by skilled analysts in order to obtain data with sufficient reproducibility.

Recently, a new method using *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) has been developed for allantoin determination in serum, lymph, urine, and plants (10–12). Some of these determinations use thin layer chromatographic separation, followed by densitometry.

Attempts at direct measurement of allantoin in electrochemical oxidation products of purine derivatives have also been carried out by using liquid chromatography (13, 14).

We have found that allantoin readily reacts with nitrobenzaldehydes in alkaline medium to give crystalline products that absorb in the ultraviolet (UV) range. In the present work, an analytical procedure is described for determining allantoin, based on high pressure liquid chromatographic (HPLC) separation of a UV-labeled allantoin derivative. This method is applied to the quantitative measurement of allantoin in some cosmetic preparations.

METHOD

Reagents

Commercially available reagent grade allantoin; *N,N*-dimethylformamide (DMF); and 1,8-diazabicyclo-(5,4,0)-7-undecene (DBU, Tokyo Kasei Kogyo Co., Ltd) were used without further purification. *p*-Nitrobenzaldehyde (*p*-NBA, Aldrich Chemical Co., Inc.) was purified by recrystallization from benzene-hexane (1 + 1). Various ingredients used for cosmetic preparations were all technical grade, unless otherwise noted.

Apparatus

(a) *Centrifuge.*—Model H-100 B2 (Kokusan Enshinki Co., Ltd).

(b) *Ultrasonic generator.*—Bransonic 220 (Yamato Scientific Co., Ltd).

(c) *Rotary vacuum evaporator.*—Model N-1 (Tokyo Rikakikai Co., Ltd).

(d) *High pressure liquid chromatograph.*—Hitachi

[1] Address correspondence to this author.
Received November 20, 1981. Accepted April 14, 1982.

0004-5756/82/6506-1362-04$01.00

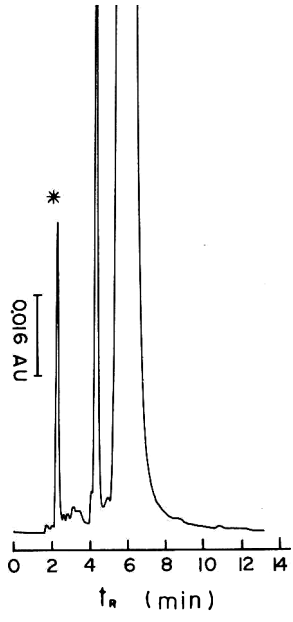


Figure 1. Typical HPLC chromatogram of allantoin in cosmetic lotion (formula shown in Table 1). * Indicates peak of allantoin-*p*-NBA reaction product.

635S (Hitachi Ltd) equipped with UV detector (UVILOG-5 III, Applied Spectroscopic Instruments Co., Ltd) and 4.6 mm id × 300 mm stainless steel column packed with octadecylsilylated silicic acid (Hitachi Gel No. 3053). Mobile phase 45% (v/v) acetonitrile in 0.01M aqueous potassium dihydrogen phosphate (pH 2.7), flow rate 1.3 mL/min. Monitor column eluates at 270 nm and maintain column at 40°C.

Derivatization and Calibration Curve

In a 10 mL volumetric flask, dissolve allantoin (3–50 mg, 0.019–0.315 mmol) in 8 mL DMF, containing 1.0 g (6.6 mmol) *p*-NBA. Carry out reaction at room temperature. After *p*-NBA dissolves, dilute solution to 10 mL with DMF. Add 10 μL DBU with stirring, let stand 30 min at 20°C.

Prepare calibration curve by using standard solutions of allantoin-*p*-NBA reaction product prepared from range of allantoin indicated.

High Pressure Liquid Chromatography

Analyze reaction product of allantoin with *p*-NBA by injecting 1 μL aliquot into HPLC system described in apparatus.

Determination of Allantoin in Cosmetic Preparations

Add 10 g cream or lotion (equivalent to 10 mg allantoin) and 20 mL absolute ethanol to 50 mL vacuum flask. Dehydrate mixture by continuous azeotropic removal of water under reduced pressure with rotary evaporator at <60°C. Add 50 mL ethyl ether to residue and ultrasonicate mixture 5 min. Then transfer contents to glass centrifuge tube and centrifuge 10 min at 5000 rpm. Carefully decant supernatant solution and dry precipitate in vacuum. Transfer residue to 10 mL volumetric flask with 8 mL DMF and carry out reaction with *p*-NBA as described above.

Results and Discussion

A typical HPLC chromatogram of allantoin-*p*-NBA reaction product in samples prepared

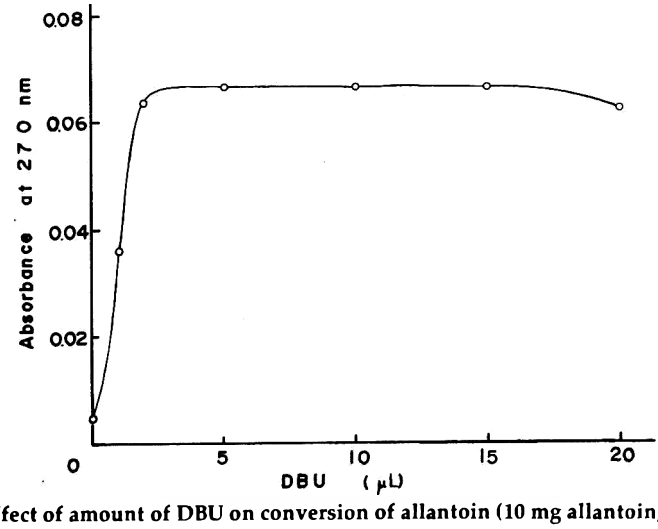


Figure 2. Effect of amount of DBU on conversion of allantoin (10 mg allantoin; 1.0 g *p*-NBA).

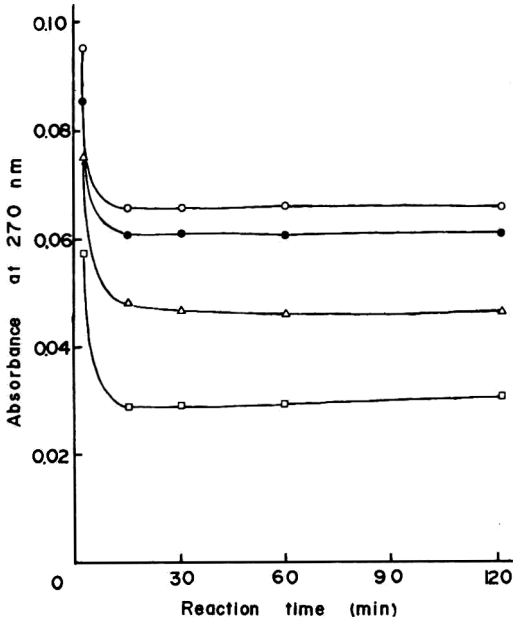


Figure 3. Time for conversion of allantoin and influence of temperature (10 mg allantoin; 1.0 g *p*-NBA; 10 μL DBU). ○, 20°C; ●, 30°C; △, 50°C; □, 80°C.

from cosmetic preparations is shown in Figure 1.

Preliminary investigation revealed that allantoin reacts readily with nitrobenzaldehydes in DMF in the presence of basic catalysts, but not in other solvents such as dimethyl sulfoxide or pyridine. Acidic catalysts, BF_3 or HCl for instance, were ineffective in this reaction. Of the basic catalysts examined (pyridine, triethylamine, DBN, and DBU), DBU was the most satisfactory, and *p*-NBA was the most reactive among 3 isomers of nitrobenzaldehyde.

The optimum quantity of DBU for 10 mg of allantoin was 5–15 μL, and 20°C was the preferred reaction temperature (Figures 2 and 3, respectively). Although the reason for the rapid decrease of allantoin conversion right after the onset of reaction is not understood, the reaction product was highly stable in the reaction medium for more than 6 h. HPLC analysis was thus carried out 30 min after the initiation. The conversion of allantoin was dependent on the amount of *p*-NBA added, and the larger the proportion of *p*-NBA to allantoin, the higher the yield. However, at least a 15-fold amount (molar ratio) of *p*-NBA relative to allantoin sufficed to achieve the detection of 10 ng allantoin, and linearity of the calibration curve. In the present reaction system, more than 20-fold *p*-NBA was used.

Table 1. Formulas of laboratory-prepared cosmetics

Ingredient	Content (%)
Lotion (Sample A)	
Ethyl alcohol	20.0
Propylene glycol	3.0
Polyoxyethylene oleyl ether (25 E.O.)	0.3
Potassium dihydrogen phosphate[a]	0.1
Bergamot oil	0.05
Allantoin	0.1
Brilliant Blue FCF	trace
Water	76.45
Cream (Sample B)	
Liquid paraffin	10.0
Cetyl alcohol	3.0
Polyoxyethylene cetyl ether (5.5 E.O.)	2.0
Sodium cetylsulfate[a]	1.0
Bergamot oil	0.1
Butyl *p*-hydroxybenzoate[a]	0.1
Methyl *p*-hydroxybenzoate[a]	0.1
Allantoin	0.1
Water	83.6

[a] Reagent grade.

Table 2. Determination of allantoin in cosmetic samples

Sample	Taken, g	Allantoin Added, %	Allantoin Rec., %[a]
A	10.0	0.1	102.4 ± 1.2
B	10.0	0.1	97.1 ± 2.0
C[b]	10.0	0.1	98.1 ± 1.1

[a] Five replicates for each mean ± SD.
[b] Lotion, commercially purchased.

The standard curve of peak heights of the reaction product was linear throughout the operational range (0.3–5 μg allantoin). The minimum amount of allantoin that was detectable at maximum sensitivity was 10 ng.

The present procedure was applied to determining allantoin in a few cosmetic preparations. Formulas of the laboratory-prepared cosmetics are given in Table 1; results are summarized in Table 2. The concentration of allantoin was 0.1%, which is similar to the 0.1–0.3% levels used in cosmetics. The small coefficient of variation values (1.1–2.1%) and high recoveries (97.1–102.4%) suggest that this method has practical applications. Further attempts at structural elucidation of the allantoin-*p*-NBA reaction product are now under way.

REFERENCES

(1) Flesch, P. (1958) *Drug Cosmet. Ind.* **83**, 168–176
(2) Matsumoto, T., Yatazawa, M., & Yamamoto, Y.

(1977) *Plant Cell Physiol.* **18,** 353–359
(3) Stahl, A., Schang, A.-M., Brogard, J.-M., & Coumaros, C. (1970) *Ann. Biol. Clin.* **28,** 377–385
(4) Pentz, E. I. (1969) *Anal. Biochem.* **27,** 333–342
(5) Young, E. G., & Conway, C. F. (1942) *J. Biol. Chem.* **142,** 839–853
(6) Borchers, R. (1977) *Anal. Biochem.* **79,** 612–613
(7) Sumi, T., Umeda, Y., Kishi, Y., Kakimoto, F., & Takahashi, K. (1979) *Anal. Biochem.* **75,** 563–567
(8) Kaito, T., Sagara, K., Ito, Y., Nakamura, K., & Anmo, T. (1977) *Yakugaku Zasshi* **97,** 165–170 (Japanese, with English summary)
(9) Christman, A. A., Foster, P. W., & Esterer, M. B. (1944) *J. Biol. Chem.* **155,** 161–171
(10) Abraham, J., Simeone, F. A., & Hopkins, R. W. (1976) *Anal. Biochem.* **70,** 377–380
(11) Herridge, D. F., Atkins, C. A., Pate, J. S., & Rainbird, R. M. (1978) *Plant Physiol.* **62,** 495–498
(12) Vrbaški, M. M., Grujić-Injac, B., & Gajić, D. (1978) *Anal. Biochem.* **91,** 304–308
(13) Cleary, M. T., & Dryhurst, G. (1977) *Anal. Chim. Acta* **94,** 343–350
(14) Owens, J. L., Thomas, H. H., & Dryhurst, G. (1978) *Anal. Chim. Acta* **96,** 89–97

SUGARS AND SUGAR PRODUCTS

High Performance Liquid Chromatographic Determination of Minor Saccharides in Corn Sugar: Collaborative Study

C. EARL ENGEL, PHILIP M. OLINGER, and RAFFAELE BERNETTI[1]
Corn Refiners' Association, 1001 Connecticut Ave, NW, Washington, DC 20036

Collaborators: K. M. Brobst; R. A. Holme; R. E. Lodge

The official first action AOAC method for quantitative determination of saccharides in corn syrup by liquid chromatography was reinvestigated to expand its scope to products containing dextrose in excess of 98%. Because of the low levels of saccharides other than dextrose (DP_1), separations are carried out only of the DP_1, the disaccharides (DP_2), and the tri- and higher saccharides (DP_{3+}) on an Aminex Q15-S, calcium form, cation exchange column. Concentrations of solids in water solvent injected into the liquid chromatograph are higher than those in the AOAC method to increase precision of analysis of the minor saccharides. All carbohydrate components in solution are eluted from the column; therefore, loss of refractive index detector linearity with respect to dextrose is compensated by use of an external maltose (DP_2) standard to correct for DP_2 and DP_{3+} response and dextrose is calculated by difference from 100. With mean values of 0.520% DP_2 and 0.244% DP_{3+}, the reproducibility and repeatability coefficients of variation are 14.6 and 6.7% for DP_2, and 19.3 and 11.9% for DP_{3+}, respectively. Dextrose levels of 99.23% are reproducible within 0.161. The coefficients of variation of the DP_2 component are lower by this method than by the AOAC method at levels up to 2% DP_2. The method has been adopted official first action.

The official AOAC method for the determination of saccharides in corn syrup by high performance liquid chromatography (HPLC) (1) has been especially designed on the basis of samples containing no more than 90% of the major saccharide component and no less than 1–2% of the minor saccharide component(s) (2). Accordingly, it specifies that a sample be diluted to about 12% dry substance before 30 μL is injected into the chromatograph. The results are either computed automatically or calculated according to the equation:

$$\% \text{ Component} = \frac{[(\text{area component})\,(\text{KF component})] \times 100}{[(\text{area}_f)\,(\text{KF}_f)] + [(\text{area}_g)\,(\text{KF}_g)] + \ldots [(\text{area}_{5+})\,(\text{KF}_{5+})]}$$

where the subscripts f, g, 2, 3, 4, and 5+, respectively, refer to fructose, glucose (dextrose), di-, tri-, tetra-, and pentasaccharides and higher saccharides. With the 2 monosaccharides designated as DP_1, the sequence is also described as DP_1, DP_2, . . . DP_{5+}.

Two problems may be encountered when the scope of the above method is expanded to corn sugar type samples which contain more than 98% dextrose and levels of other saccharides lower than 1%, most often in the range 0.1–0.5%: The linearity of the refractive index detector is exceeded with respect to the major component dextrose; and the peaks of the chromatogram corresponding to the minor saccharides may be difficult to integrate to baseline. Correcting the second problem by injecting higher solids only worsens the first problem, and the normalized area quantitations calculated according to the equation above yield lower and lower dextrose results. These problems and their potential solution by calculating on the basis of an external minor saccharide standard were illustrated by H. D. Scobell (A. E. Staley Mfg Co., Decatur, IL, 1978) and are shown in Table 1, where the same sample of corn sugar was injected at 3 solids levels and the calculations were performed according to the official AOAC method and by an external maltose standard; in the latter calculation, dextrose is obtained by difference of the minor saccharide total from 100.

This paper describes the modifications of the AOAC liquid chromatographic method to make it applicable to corn sugar and reports the collaborative data obtained on 6 samples containing from 98 to 100% dextrose.

Received November 9, 1981. Accepted April 9, 1982.
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.* **66**, March issue (1983).

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

[1] Present address: CPC International, Inc., Corn Products, Moffett Technical Center, PO Box 345, Argo, IL 60501. Address correspondence to this author.

0004-5756/82/6506-1366-04$01.00

Table 1. Effect of method of calculation on results of corn sugar analysis

Sample injected[a]	Area normalization quantitation				External standard quantitation			
	Dextrose, %	DP_2, %	DP_3, %	DP_{4+}, %	Dextrose, %	DP_2, %	DP_3, %	DP_{4+}, %
3.0	99.63	0.21	0.02	0.12	99.42	0.32	0.03	0.23
6.0	99.41	0.33	0.04	0.22	99.39	0.32	0.04	0.25
10.0	99.12	0.50	0.06	0.32	99.36	0.35	0.04	0.25

[a] mg Disaccharide.

Minor Saccharides in Corn Sugar by Liquid Chromatography – Official First Action

Principle

Polysaccharides in high (98 + %) dextrose corn sugar are quantitated vs maltose std and calcd as disaccharides (DP_2) and tri- and higher saccharides (DP_{3+}).

Determination

Proceed as in **31.229-31.236,** except use only Aminex Q15-S resin column (**31.229**(g)), or equiv., and use ca 3 g maltose.H_2O std/25 mL instead of mixed sugar std **31.230**(e). Compute dry basis concn of maltose by multiplying by 0.94. Det. response factor for maltose dry basis by injecting 10 μL std soln and proceeding as in **31.233.** Prep. sample as in **31.234**. Complete detn as in **31.235,** but inject 50 μL dild sample.

Calculations

Results are computed automatically when using **31.229**(c). List DP_2 results and combine all tri- and higher saccharides and list sum as DP_{3+}. Subtract sum of DP_2 and DP_{3+} from 100 to obtain glucose (dextrose) (DP_1) value. Report results in ash-free, carbohydrate dry substance basis. In absence of computing integrator, list areas for DP_2 and sum for DP_{3+}, multiply each by maltose response factor and by 100 to obtain $\%DP_2$ and $\%DP_{3+}$. Subtract sum of $\%DP_2$ and $\%DP_{3+}$ from 100 to obtain $\%DP_1$.

Collaborative Study

Six samples of corn sugar dextrose were sent to 5 collaborators. Samples A and B were anhydrous, and Samples C, D, E, and F were dextrose monohydrate. All 6 samples represented different commercial production batches. The maltose standards were prepared by each collaborator. Collaborators were asked to perform duplicate liquid chromatographic analysis on the samples and to report DP_2 and DP_{3+}, using maltose external standard in the calculations, and to report glucose (dextrose) by difference of the sum of the minor saccharides from 100. All collaborators followed the recommended procedure and used Aminex Q15-S.

Results and Recommendations

Figure 1 is a typical chromatogram, showing the performance of a commercially prepacked Aminex HPX 87 Ca^{++} column, equivalent to the Aminex Q15-S resin columns packed individually in the collaborators' laboratories.[1] Tables 2–4 list the collaborative results obtained for glucose, DP_2, and DP_{3+}, respectively. Results evaluated statistically according to Steiner (3) showed no outlying laboratories with respect to ranking. All Dixon outliers were included in the

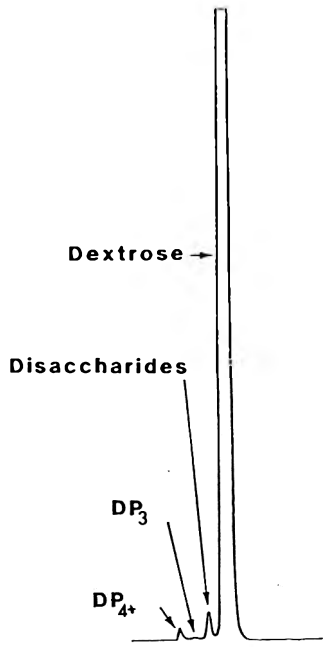


Figure 1. Performance of prepacked column in determination of corn sugar.

[1] The convenience feature of prepacked columns was not available in 1979 when the collaborative study was performed.

Table 2. Collaborative results for glucose (%) in corn sugar by liquid chromatography

	Sample						
Coll.	A	B	C	D	E	F	All samples
1	99.62	99.50	99.62	99.20	99.09	98.12	
	99.61	99.57	99.72	99.15	99.19	98.27	
2	99.67	99.54	99.60	99.08	99.07	97.92	
	99.71	99.49	99.59	99.02	99.04	97.87	
3	99.77	99.59	99.73	99.27	99.17	98.27	
	99.79	99.69	99.60	99.25	99.20	98.32	
4	99.73	99.59	99.41[a]	99.26	99.30	97.96	
	99.76	99.48	99.37[a]	99.14	99.40	98.05	
5	99.76	99.60	99.66	99.20	99.20	98.32	
	99.73	99.60	99.69	99.20	99.18	98.31	
Mean	99.72	99.56	99.60	99.18	99.18	98.14	99.23
Reproducibility	0.066	0.065	0.127	0.084	0.113	0.189	0.161
CV, %	0.066	0.065	0.127	0.085	0.114	0.192	0.162
Repeatability	0.019	0.054	0.054	0.046	0.047	0.060	0.049
CV, %	0.019	0.054	0.054	0.046	0.047	0.061	0.049

[a] Outlier.

Table 3. Collaborative results for total DP_2 (%) in corn sugar by liquid chromatography

	Sample						
Coll.	A	B	C	D	E	F	All samples
1	0.20	0.44	0.28	0.47	0.70	1.28	
	0.24	0.34	0.22	0.49	0.59	1.11	
2	0.21	0.37	0.30	0.53	0.69	1.33	
	0.19	0.40	0.31	0.55	0.70	1.40	
3	0.15	0.29	0.23	0.42	0.59	1.08	
	0.12	0.29	0.29	0.43	0.58	1.11	
4	0.21	0.37	0.44[a]	0.49	0.52	1.28	
	0.19	0.39	0.44[a]	0.49	0.50	1.22	
5	0.16	0.32	0.26	0.46	0.60	1.06	
	0.17	0.32	0.24	0.45	0.61	1.07	
Mean	0.184	0.353	0.301	0.478	0.608	1.194	0.520
Reproducibility	0.036	0.051	0.083	0.043	0.073	0.129	0.076
CV, %	19.6	14.4	27.6	9.0	12.0	10.8	14.6
Repeatability	0.018	0.034	0.028	0.010	0.036	0.062	0.035
CV, %	9.8	9.6	9.3	2.09	5.9	5.2	6.7

[a] Outlier.

Table 4. Collaborative results for DP_{3+} (%) in corn sugar by liquid chromatography

	Sample						
Coll.	A	B	C	D	E	F	All samples
1	0.18	0.06	0.10	0.33	0.19	0.60	
	0.15	0.09	0.06	0.36	0.24	0.62	
2	0.12	0.09	0.10	0.39	0.24	0.69	
	0.10	0.11	0.10	0.43	0.26	0.67	
3	0.08	0.12	0.04	0.31	0.24	0.65	
	0.09	0.02	0.11	0.32	0.22	0.57	
4	0.06	0.04	0.15[a]	0.25	0.18	0.73	
	0.05	0.13	0.19[a]	0.37	0.10	0.68	
5	0.08	0.08	0.08	0.34	0.20	0.54	
	0.10	0.08	0.07	0.35	0.21	0.54	
Mean	0.101	0.082	0.100	0.345	0.208	0.629	0.244
Reproducibility	0.042	0.044	0.045	0.049	0.047	0.069	0.047
CV, %	41.6	53.7	45.0	14.2	22.6	11.0	19.3
Repeatability	0.013	0.044	0.029	0.041	0.031	0.031	0.029
CV, %	12.9	53.7	29.0	11.9	14.9	4.9	11.9

[a] Outlier.

statistical evaluation to maintain the integrity of the data.

Reproducibilities and repeatabilities with respect to analyses of DP_2 and DP_{3+} were quite acceptable, considering the respective low levels, and were better than corresponding, earlier results on high fructose corn syrups (2). The glucose (dextrose) results were reproducible within 0.16% and repeatable within 0.05%. The high coefficients of variation for the DP_{3+} components still reflected reproducibilities within 0.04% at saccharide levels of 0.1–0.2%. It is recommended that the method, as presented, be adopted official first action.

Acknowledgments

The Associate Referee thanks other members of the Analytical Procedures Committee of the Corn Refiners Association, Inc., who participated in this study, as follows:

K. M. Brobst, A. E. Staley Manufacturing Co., Decatur, IL

R. A. Holme, Amstar Corp., Dimmitt, TX

R. E. Lodge, The Hubinger Co., Keokuk, IA

P. M. Olinger, Clinton Corn, Clinton, IA

The Associate Referee laboratory participated on behalf of CPC International, Inc., Argo, IL.

References

(1) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs. **31.228-31.236**

(2) Engel, C. E., & Olinger, P. M.(1979) *J. Assoc. Off. Anal. Chem.* **62,** 527–532

(3) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA, pp. 72–80

INDUSTRIAL CHEMICALS

Volatile Organic Compounds in Water at Thirty Canadian Potable Water Treatment Facilities

REIN OTSON, DAVID T. WILLIAMS, and PETER D. BOTHWELL
Health and Welfare Canada, Bureau of Chemical Hazards, Health Protection Branch, Environmental Health Centre, Tunney's Pasture, Ottawa, Ontario, Canada, K1A 0L2

A static headspace technique and previously developed and evaluated dynamic headspace and liquid-liquid extraction techniques were applied in a survey of volatile organic compounds in water at 30 Canadian potable water treatment facilities. Of the 43 compounds investigated, 27 compounds were detected in the water samples. Chloroform and bromodichloromethane occurred most frequently in treated water, and trichloroethylene, tetrachloroethylene, 1,2-dichloroethane, dichloromethane, benzene, toluene, ethylbenzene, and the xylenes occurred frequently in both treated and raw water. Only chloroform occurred frequently at levels above 10 μg/L in treated water. Generally, higher levels of organic compounds were found during August-September than during November-December.

Recently, a wide variety of organic compounds has been identified in raw and potable water supplies (1, 2). Some of these compounds may pose a threat to human health and therefore are of immediate concern (3–5). In Canada, guidelines (4, 5) have been established for maximum acceptable concentrations, and national (6–9) and provincial (10–12) surveys have been conducted to obtain information on levels of organic compounds in potable water. However, trihalomethanes were the only volatile organic compounds determined during a previous Canadian nationwide survey (6).

This paper presents the results of a survey of a broad spectrum of volatile organic compounds in raw and treated water at 30 Canadian potable water treatment facilities serving about 5.5 million consumers across Canada. Suitable analytical techniques for identification and quantitation of compounds with boiling points ranging up to about 200°C were required for this survey. The development and evaluation of a liquid-liquid extraction (LLE) technique (13) and a dynamic headspace (DHS) technique (14) are reported elsewhere. A static headspace (SHS) technique was used for determination of very volatile organic compounds which could not be determined by the LLE and DHS techniques.

Received October 8, 1981. Accepted March 23, 1982.

Experimental

Equipment and Reagents

Reagents, equipment, and operating conditions for liquid-liquid extraction (LLE) technique and dynamic headspace (DHS) technique analysis have been described (13, 14).

For DHS analysis by gas chromatography-mass spectrometry (GC/MS-DHS), the following equipment was used:

(a) *Liquid sample concentrator.*—Tekmar Model LSC-1 (15) equipped with stainless steel traps (20 cm × 2.2 mm id) packed with 110 ± 5 mg 60–80 mesh Tenax GC, and tube-needle interface to GC injection port of GC/MS. Operating conditions: water sample (5.0 mL) at 40°C, purged 15 min at nitrogen UHP flow rate 60 mL/min; trap desorbed at 200°C for 10 min at nitrogen UHP flow rate 30 mL/min; trap baked at 200°C for 60 min at nitrogen UHP flow rate 20 mL/min.

(b) *Gas chromatograph–mass spectrometer (GC/MS).*—Finnigan Model 4000 coupled with Series 6000 data system and equipped with glass column (1.8 m × 2.0 mm id) packed with 60–80 mesh Tenax GC. Operating conditions: helium UHP flow rate 0 mL/min during Model LSC-1 trap desorb and 20 mL/min at other times; injection port 200°C; column 35°C during trap desorb, then programmed at 20°/min to 100°C and at 8°/min to 180°C, and held 25 min; separator and ion source 250°C.

For static headspace (SHS) technique analysis, the same equipment was used as for the DHS technique, except for the following:

(c) *GC column.*—Same Tenax column as for DHS. Operating conditions: initial temperature at 50°C held 0.5 min, programmed at 20°/min to 180°C, and held 5 min.

(d) *Syringe.*—Hamilton Model 1002 TLLCH gas-tight syringe with KF-723 needle.

(e) *Standard solutions.*—Aqueous standard solutions prepared as described elsewhere (13, 14) were used for LLE, DHS, and GC/MS-DHS techniques. Single component and composite aqueous standard solutions containing compounds which had not been investigated previ-

0004-5756/82/6506-1370–05$01.00

ously (13, 14), were prepared in the same manner.

Sampling Details

Twenty-nine municipalities (30 treatment plants), serving about 5.5 million consumers and including all large centers of population, were selected across Canada. At each treatment plant, water was allowed to flow from a tap for several minutes before grab samples were collected and immediately sealed, headspace-free, in amber glass bottles (13). Bottles were shipped within 24 h to a central laboratory (Health and Welfare Canada) where they were stored at 4°C until analysis. For the first set of samples obtained during August–September 1979, raw water and plant effluent water samples were collected within 1 h in one day, and an additional plant effluent water sample was collected 24 h later. Duplicate samples of raw and plant effluent water were collected for the second set of samples during resampling in November–December 1979.

Analytical Procedures

A 40 mL aqueous aliquot was withdrawn from a full, 120 mL capacity, amber glass bottle and analyzed by the DHS technique. The container was then inverted and stored at 4°C for ≤3 weeks. Before SHS analysis, the inverted bottle was left at ambient temperature (22–25°C) overnight and then set upright in a water bath at 25 ± 1°C for 1 h. A 2.0 mL headspace aliquot was then withdrawn from the sealed bottle for analysis. Within 1 h, 3.0 mL hexane was added to the sealed bottle contents (80 mL) and the container was stored at ambient temperature for 4–7 days. An aliquot of about 1.5 mL of the hexane layer was then transferred and sealed into an autosampler vial which was stored for several weeks at 4°C before analysis (13) of the contents. For some survey samples and aqueous standard solutions, a 5.0 mL aqueous aliquot was analyzed by the GC/MS-DHS technique before the LLE technique analysis step.

Purified water (14) blanks and aqueous standard solutions, containing 25 or 100 μg of chloroform/L and 4 μg/L of the other compounds, were regularly prepared (14) and stored with each group of survey samples on the day when the latter were received at the central laboratory. Frequently, additional aqueous standard solutions containing the compounds at concentrations of 1, 16, and 50 μg/L were also prepared and analyzed. Survey samples and standard solutions were analyzed together in groups throughout the analytical sequence to allow for the effect of storage and handling during quantitation. Also, survey sample analyses were often done concurrent with the evaluation tests for the analytical techniques (13, 14). Analytical results for triplicate sets of aqueous standard solutions were used to evaluate the SHS technique. The quantitation limits used or listed in these studies were greater than the detection limits and represent concentrations at or above which acceptable analytical precision could be contained.

Results and Discussion

The SHS technique was used for the determination of some very volatile alkyl halides which could not be determined conveniently by the LLE and DHS techniques. Retention times and quantitation limits for some volatile halogenated hydrocarbons, methanol, and acetone are listed in Table 1. Analyses of replicate solutions generally showed peak area precision values of <20% RSD. However, storage of aqueous standard solutions for several days often resulted in poor analytical precision and loss of very volatile organic compounds from the solutions was particularly noticeable if the containers were stored at room temperature. It was concluded that, for the water samples from the survey, analytical values obtained by the SHS technique for the first 7 compounds listed in Table 1 should be regarded as semiquantitative. Methanol and acetone would only interfere with the FID determination of co-eluting alkyl halides (Table 1) if the former compounds occur at concentrations near or above 1 mg/L in water. Many of the compounds eluting after chloroform showed detection limits of 10 μg/L or better, but the chromatographic conditions were not optimized for their determination.

It has been demonstrated (13, 14) that bottled aqueous standard solutions can be stored for several weeks without significant effect on the quantitation of compounds determined by the LLE and DHS techniques. Nevertheless, to ensure reliable quantitation in the present study, standard solutions were prepared, stored, and analyzed at times corresponding to water sample collection, storage, and analysis. Identical manipulation of samples and standard solutions was also important, because 80 mL aqueous aliquots instead of the suggested (13) 120 mL aliquots were used for LLE analysis. It was determined that storage of the extractant in contact with the aqueous phase for 4–7 days provided about the same extraction recoveries as the procedure

Table 1. Typical SHS technique results for volatile organic compounds in water

Compound	Retention time, min	Quant. limit, μg/L[a]	
		FID	HECD
CCl_2F_2	1.15	10	50
CH_3Cl	1.61	5	50
CH_2CHCl	2.19	1	20
CH_3Br	2.63	5	40
CH_3CH_2Cl	2.97	5	20
CH_2CCl_2	3.80	5	10
CH_2Cl_2	3.92	2	5
CH_3OH	1.74	~1000	—
CH_3COCH_3	3.89	~1000	—
CHClCHCl	4.31	1	1
$CHCl_2CH_3$	4.66	1	2
$CHCl_3$	4.97	1	1
CH_2ClCH_2Cl	—	1	1
CCl_2CHCl	5.88	1	2

[a] See text.

applying 24 h of agitation (13). Detection limits obtained by the LLE technique did not suffer significantly from the changes in procedure. Peak area precision was generally better than 10% RSD for both LLE and DHS analyses of samples and standard solutions at concentrations ranging from 5 to 100 μg/L. The GC/MS-DHS technique was not evaluated for precision, detection limits, or linearity of the detector response-concentration relationship. Although quantitative results were estimated by comparison of the mass spectrometer response for compounds in survey samples and in aqueous standard solutions, the technique was applied chiefly for confirmation of compound identification by the other techniques.

The occurrence and levels of the 43 compounds summarized in Tables 2 and 3 were determined by DHS, SHS, and LLE technique analyses of the collected water samples. Usually more than one technique or detector was available (13, 14) to determine the occurrence of a compound. Results for analyses obtained by techniques-detectors listed in Table 3 were judged most reliable and were used for quantitation. The quantitation techniques were chosen after identification of, and to minimize interference by, compounds other than the one being quantitated. GC/MS-DHS analysis of 14 selected water samples confirmed the analytical results obtained by the 3 other techniques, and no new contaminants were discovered.

Table 2. Compounds investigated but not detected in water

Compound	Quant. limit, μg/L[a]
CH_3Br	5
CH_3CH_2Cl	5
CH_3OH	~1000
CH_3COCH_3	~1000
CCl_3CH_3	5
CCl_4	5
$CH_2ClCH_2OCHCH_2$	50
$CHCl_2CClCHCl$	1
o-$C_6H_4Cl_2$	1
$(CH_2ClCH_2)_2O$	1
CCl_3CCl_3	1
$CCl_2CClCHCl_2$	1
$C_6H_5NO_2$	5
$CCl_2CClCClCCl_2$	1
1,2,4-$C_6H_3Cl_3$	1
$C_{10}H_8$	1

[a] See text.

Sixteen of the 43 compounds investigated could not be detected in any water samples at the levels shown in Table 2. Each of the remaining 27 compounds was found at least in one sample and the analytical results for these compounds are summarized in Table 3. Of all the compounds, chloroform and bromodichloromethane, which may be formed (16) during the chlorination process, occurred most frequently in treated water. Detectable levels of the other 2 trihalomethanes, chlorodibromomethane and bromoform, were not found as frequently. The occurrence of trihalomethanes in some raw water samples may be due to contamination by treated water from system backwashing at the time of sampling. Trichloroethylene, benzene, toluene, tetrachloroethylene, dichloromethane, 1,2-dichloroethane, and the C_8H_{10} isomers, ethylbenzene and the xylenes, occurred frequently in both treated and raw water. The isomers are listed in pairs in Table 3 since they could not be resolved under the DHS technique GC conditions and could not be detected at levels below 5 μg/L by the LLE technique.

Table 3. Mean ($\bar{x}$) and maximum (Max.) levels (μg/L) and frequency (f) of detection of compounds found in water

Compound[a]	Quant. technique[b]	August–September									November–December					
		Raw			Treated (day 1)			Treated (day 2)			Raw			Treated		
		$\bar{x}$	Max.	f	$\bar{x}$	Max.	f	$\bar{x}$	Max.	f	$\bar{x}$	Max.	f	$\bar{x}$	Max.	f
CCl_2F_2	SHS-FID			0			0			0	<10	<10	1	<10	<10	1
CH_3Cl	SHS-FID			0			0			0	<5	<5	1	5	<5	2
CH_2CHCl	SHS-FID			0			0			0	<1	<1	1	<1	<1	1
CH_2CCl_2	SHS-FID			0			0	<1	~20	1			0			0
CH_2Cl_2(12)	SHS-HECD	~6	~50	9	~10	~50	15	~10	~50	16	<1	12	9	~3	~50	9
CHClCHCl	DHS-HECD	1	23	2	3	26	4	3	20	6			0	1	32	1
$CHCl_2CH_3$	DHS-FID	<1	<1	2	<1	<1	2	<1	<1	3			0	<1	<1	2
$CHCl_3$(6,11,12)	DHS-HECD	5	60	15	37	100	30	35	110	29	2	13	13	21	60	28
CH_2ClCH_2Cl	DHS-HECD	2	33	4	4	30	10	5	29	11	<1	11	6	<1	10	4
C_6H_6(12)	DHS-FID	2	48	19	3	47	23	2	15	15	<1	2	13	<1	8	17
CCl_2CHCl(11,12)	LLE-ECD	1	2	24	<1	3	16	1	9	14	2	9	24	<1	5	21
$CHBrCl_2$(6,11,12)	DHS-HECD	<1	13	2	3	16	24	3	16	24	<1	<1	2	2	12	25
$CH_2ClCHClCH_3$	DHS-HECD			0	<1	<1	1			0			0			0
$CHClCHCH_2Cl$	DHS-HECD			0			0			0	<1	<1	1	<1	<1	2
$CHCl_2CH_2Cl$	DHS-FID			0			0	<1	7	2			0			0
$C_6H_5CH_3$(12)	DHS-FID	<1	2	15	2	27	20	2	14	10	<1	1	12	<1	13	16
CCl_2CCl_2	LLE-ECD	<1	2	9	<1	2	11	<1	4	13	<1	1	15	<1	2	15
$CHBr_2Cl$(6,11,12)	DHS-HECD	<1	<1	1	<1	4	9	<1	4	7			0	<1	3	6
C_6H_5Cl	DHS-HECD	<1	<1	3	<1	5	9	<1	2	3			0	<1	<1	4
$C_6H_5(C_2H_5)$ (12)	DHS-FID	<1	<1	7	<1	10	9	<1	7	6	<1	2	7	<1	5	6
p-$C_6H_5(CH_3)_2$(12)																
m-$C_6H_4(CH_3)_2$ (12)	DHS-FID	<1	<1	2	<1	8	8	<1	7	8	<1	<1	5	<1	2	6
o-$C_6H_4(CH_3)_2$(12)																
$CHBr_3$(6)	LLE-ECD			0	<1	2	4	<1	1	3			0	<1	1	1
$CHCl_2CHCl_2$(12)	LLE-HECD			0			0	<1	1	1	<1	12	1			0
m-$C_6H_4Cl_2$(12)	DHS-FID	<1	<1	1	<1	1	1			0			0			0
p-$C_6H_4Cl_2$(12)	DHS-FID	<1	<1	5	<1	<1	6			0			0			0
Total with THM		~18		120	~66		202	~65		172	~7		111	~32		167
Total without THM		~13		102	~25		135	~26		109	~5		96	~9		107

[a] Numbers in parentheses identify literature references of compounds previously reported in Canadian potable water.
[b] Technique–detector.

Compounds which have previously (6, 11, 12) been detected in Canadian potable water are noted in Table 3 and generally were those most frequently found in the present survey. Carbon tetrachloride, hexane, and C_9H_{12} and $C_{10}H_{14}$ substituted benzene isomers were previously (11, 12) found but were not detected in this survey, possibly due to unfavorable chromatographic conditions. The previous studies did not show the occurrence of 12 of the infrequently detected compounds listed in Table 3.

For 7 compounds of the 27 compounds detected, chloroform, bromodichloromethane, dichloromethane, 1,2-dichloroethane, 1,2-dichloroethylene, benzene, and toluene, the mean concentration in treated water at the 30 treatment facilities was >1 μg/L. Trichloroethylene was found at a mean concentration exceeding 1 μg/L in raw water during November–December. The median concentration (not shown) in raw or treated water was <1 μg/L for all compounds listed in Table 3, except chloroform, bromodichloromethane, benzene, and trichloroethylene. The median values for these 4 compounds were generally lower than the corresponding mean values. These results show that, although 27 compounds were detected in raw and treated water, few were regularly found at levels exceeding 1 μg/L and only chloroform occurred frequently at levels above 10 μg/L in treated water. Although the quantitation of the first 7 compounds listed in Table 1 was uncertain, results also showed that their concentration in a water sample can decrease quite rapidly over 24 h. Also, since no significant unidentified peaks were observed during the analysis, compounds similar in structure, volatility, and detector response to those investigated were probably not present at significant levels in the water samples.

Generally, as shown in Table 3, the occurrence (total with THM) of organic compounds in potable water was more frequent and at higher total concentration than in raw water. When THM values were excluded from the totals (total without THM), this difference was not quite as evident. Although it is recognized that THM forms during chlorination of treated water (16), the apparent increase in levels of compounds such as benzene, toluene, 1,2-dichloroethylene, and 1,2-dichloroethane in treated water cannot be readily explained. Contamination of the water by the treatment processes and instability of some compounds in biologically active raw water are possible explanations. The totals in Table 3, which were estimated directly from analytical measurements, also demonstrated seasonal differences in mean concentration. The totals, excluding THM values, were 13 and 5 μg/L, respectively, for August–September and November–December raw water, and were 25 and 9 μg/L, respectively, for corresponding treated water samples. The observed decrease in trihalomethane levels from summer to winter has been reported in other studies (6, 17). Values obtained for trihalomethanes were well below the Canadian drinking water guidelines (5) and were in the same range of concentration as found in an earlier study (6).

Acknowledgments

We gratefully acknowledge the assistance of IEC International Environmental Consultants and Guy LeBel in the collection of water samples, and mass spectrometry services provided by Ron O'Grady. The manuscript was reviewed in-house by Valerie M. Douglas and Veluppillai Paramasigamani and was typed by Jean Ireland.

References

(1) Shakelford, W. M., & Keith, L. H. (1976) U.S. Environmental Protection Agency Report EPA-600/4-76-062
(2) Fielding, M., Gibson, T. M., James H A., McLoughlin, K., & Steel, C. P. (1981) WRC Environmental Protection Technical Report TR159
(3) Keith, L. H., & Telliard, W. A. (1979) *Environ. Sci. Technol.* **13**, 416–423
(4) Health and Welfare Canada (1978) Guidelines for Canadian Drinking Water Quality 1978
(5) *Canada Gazette* (1979) Part I, 7365–7370
(6) Williams, D. T., Otson, R., Bothwell, P. D., Murphy, K. L., & Robertson, J. L. (1980) in *Hydrocarbons and Halogenated Hydrocarbons in the Aquatic Environment*, B. K. Afghan, & D. Mackay (Eds), Plenum Press, New York, NY, pp. 503–512
(7) Williams, D. T., Benoit, F. M., McNeil, E. E., & Otson, R. (1978) *Pestic Monit. J.* **12**, 163
(8) Williams, D. T., & LeBel, G. L. (1981) *Bull. Environ. Contam. Toxicol.* **27**, 450–457
(9) Malaiyandi, M., Williams, D. T., & O'Grady, R. (1979) *Environ. Sci. Technol.* **13**, 59–62.
(10) Benoit, F. M., LeBel, G. L., & Williams, D. T. (1979) *Bull. Environ. Contam. Toxicol.* **23**, 744–778
(11) Fast, D. A., & McDonald, R. A. (1978) Saskatchewan Dept Environment Report WPB 21
(12) Smillie, R. D., Sakuma, T., & Duholke, W. K. (1978) *J. Environ. Sci. Health* **A13**, 187–197
(13) Otson, R., & Williams, D. T. (1981) *J. Chromatogr.* **212**, 187–197
(14) Otson, R., & Williams, D. T. (1982) *Anal. Chem.*, **54**, 942–946
(15) Grote, J. O. (1975) *Am. Lab.* **7**, 47–50
(16) Rook, J. J. (1974) *Water Treat. Exam.* **23**, 234–243
(17) Otson, R., Williams, D. T., Bothwell, P. D., & Quon, T. (1981) *Environ. Sci. Technol.* **15**, 1075–1080

Simple Paper Chromatographic Method for Rapid Detection of Chromate(VI) in Fresh Water

SATRAM RAJENDRABABU and NANGUNERI V. NANDA KUMAR[1]
Sri Venkateswara University, Department of Zoology, Tirupati-517502, India

A simple concentration method for detection of potassium (di)chromate directly in fresh water is described. Interference from acidity, alkalinity, or the presence of salt is eliminated by neutralization with phosphate buffer. A simple and sensitive method for potassium chromate or dichromate(VI) separation and detection by paper chromatography is also described. Succinate dehydrogenase inhibition with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT)-phenazonium methosulfate (PMS)-sodium succinate mixture as the chromogenic reagent is the detection technique. An acetone-water-EDTA solvent system allows enzymatic detection of potassium (di)chromate in fresh water with the least cleanup effort: 2 μg can be detected by a spot test or 5 μg may be chromatographically detected directly from fresh water.

Thin layer chromatographic and paper chromatographic enzymatic methods of analysis are simple, sensitive, and specific for separating and detecting heavy metal compounds like copper, mercury, cadmium, and silver (1–3). Methods reported previously for these compounds were based on a concentration technique involving precipitation by buffer treatment, evaporation by heating, chromatographic separation with 0.1% sodium chloride solution, and detection by succinate dehydrogenase inhibition. The precipitation-concentration technique and the 0.1% sodium chloride solvent system were unsuitable for the chromatographic detection of hexavalent chromate. In the present investigation, 10 solvent systems were studied for suitability for paper chromatographic separation of chromate(VI).

Earlier reports (2, 3) mention the inhibition by acid or alkaline conditions of water on the enzymatic method of estimation, so we neutralized the acidity or alkalinity with buffer. Fresh water fortified with different concentrations of acid or base is also analyzed by the paper chromatographic method. Detection or determination by non-enzymatic methods involving chromatography, volumetric analysis, or colorimetry (4–7) were either less sensitive or more cumbersome. Atomic absorption spectrophotometry is ideal for detection and estimation of heavy metals (8), but it requires a pretreatment step and its use is often limited by non-availability and cost of equipment. The present method is simple and sensitive, and permits detection under field conditions.

METHOD

Apparatus and Reagents

Use analytical grade reagents.

(a) *Potassium (di)chromate.*—Dissolve 50 mg potassium chromate or dichromate (British Drug Houses, Bombay, India) in 100 mL distilled water (500 ng/μL). Use as standard solution on Whatman No. 3 filter paper strips. Also dissolve 50 mg potassium (di)chromate in 1 L fresh water (50 ppm). Use as sample. Concentrate to 100 mL in 50°C hot air oven.

(b) *2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT)-phenazonium methosulfate (PMS)-sodium succinate.*—Prepare sodium succinate (British Drug Houses) 2.5%, INT (British Drug Houses) 0.4%, phenazonium methosulfate (British Drug Houses) 0.1% in pH 7.4 sodium phosphate buffer (0.4M) (9). Mix 5 + 5 + 2.

(c) *Enzyme preparation.*—Prepare 2.5% pig liver acetone powder (Sigma Chemical Co., St. Louis, MO) enzyme suspension or 15% fresh sheep liver homogenate in cold pH 7.4 buffer and filter through 4 layers of cheese cloth. Use filtrate as enzyme source immediately (1–3).

(d) *Whatman No. 3 filter paper strips.*—Cut 7.5 × 2.5 cm strips for paper chromatography.

(e) *Solvent system for paper chromatography.*—Mix acetone, water, and 5% EDTA (ethylenediaminetetraacetic acid) 75 + 20 + 5.

(f) *Sodium chloride solvent.*—Prepare 0.1, 0.5, and 1% in distilled water.

(g) *Potassium chromate in alkaline or acid solutions.*—Prepare 50 ppm potassium (di)chromate solution in 0.01N HCl and 0.01N NaOH.

Preparation of Samples

Prepare 50 ppm potassium chromate in fresh water, 0.01N acidic sample, or alkaline sample. Evaporate 100 mL to 10 mL in petri dish. Take

[1] Address correspondence to this author.
Received November 12, 1981. Accepted March 23, 1982.

0004-5756/82/6506-1375-04$01.00

Table 1. Appearance of paper chromatogram and mobility of 5 μg potassium (di)chromate in different solvent systems

Solvent system	Nature and appearance of chromatogram	R_f [a]
Water	widespread and diffuse at solvent front	1.0 ± 0.05
1% EDTA	trailing	—
5% EDTA	trailing	—
10% EDTA	white inhibition zone, noncompact	—
Water–hexane (50 + 50)	white inhibition zone, no mobility	—
Acetone	white zone, no mobility	—
Acetone–water (50 + 50)	fast mobility, widespread at solvent front	1.0 ± 0.05
Acetone–water (75 + 25)	fast mobility, widespread at solvent front	1.0 ± 0.05
Acetone–water–EDTA (50 + 25 + 25)	clear white zone, not compact	0.7 ± 0.035
Acetone–water–EDTA (75 + 20 + 5)[b]	compact and clear	0.75 ± 0.031
1% NaCl	widespread at solvent front, diffused, unidentifiable	—

[a] Values are mean of 6 observations ± SD.
[b] Recommended solvent system.

1 mL concentrate and add different proportions of pH 7.4 buffer (1:1, 1:2, 1:3).

Paper Chromatography

Apply 10 μL fresh water concentrate or 20–30 μL acidic or alkaline concentrate after buffer treatment (volume of application is proportional to dilution by buffer) on separate Whatman No. 3 filter strips, 2 cm above base, with graduated micro capillary. Minimize spot spreading while applying by frequently blowing with hair dryer. Place strips in different solvent system(s) mentioned in Table 1. Develop paper strips to 7 cm by ascending chromatography by hanging strips in 12 × 4 cm glass jar (1, 2). Remove strips and air-dry. Spray pig liver acetone powder enzyme suspension or freshly prepared sheep liver homogenate with a glass sprayer as fine mist until just wetting paper strips. Avoid leaching excess enzyme (1, 2). Place paper strips on clean plate and place in 40°C hot air oven 5 min. Remove strips, spray with sodium succinate–INT–PMS–buffer mixture, and replace in 70°C hot air oven. Observe white spots of potassium (di)chromate compound against pink background, following enzyme–chromogenic treatment (1, 3). Mark white chromatogram immediately, and calculate R_f values.

Results and Discussion

Potassium (di)chromate chromatographically separated with acetone–water–EDTA (75 + 20 + 5) gives a compact chromatogram visualized as a white inhibition zone at R_f 0.75. This solvent system was preferred to other systems tried (Table 1) because the presence of 5% EDTA (5 mL) improved separation of potassium (di)chromate. This might be due to binding of potassium chromate by EDTA, thereby minimizing diffusion; in the absence of EDTA, other solvent systems give poor resolution and potassium chromate was observed as a white streak at the solvent front (Table 1). The citric acid treatment previously recommended (2) for copper sulfate, cadmium sulfate, mercuric chloride, and silver nitrate is not necessary because potassium chromate is highly soluble in water, and did not adhere to the glass walls of the container.

The method was also tested for potassium (di)chromate in 0.01N acidic or alkaline water samples because industrial effluents show acidity or alkalinity. The effluent concentrations can be adjusted to 0.01N (Table 2) by dilution and titrimetry before enzymatic analysis. For 0.01N solutions, 0.04M phosphate buffer of pH 7.4 in the ratio of 1:1 to 1:2 (concentrate:buffer) is recommended for neutralization. We applied 30 μL (the maximum amount of sample or sample concentrate that can be applied) 0.01N acidic or alkaline water without potassium chromate to paper to test its interference with the enzymatic method. More than 5 μL of 0.01N HCl solution inhibited the enzyme, and a white inhibition zone was observed at the solvent edge (R_f 1). However, this R_f is different from that for potassium chromate (R_f 0.75). When 0.01N HCl was neutralized in the ratio of 1:1 with pH 7.4 phosphate buffer, no inhibition at R_f 1 was observed. The 0.01N NaOH solution had no effect before or after neutralization; in fact, the enzyme system exhibits optimal sensitivity at pH 8.4 (9). For this reason, enzyme, sodium succinate, INT, and PMS mixture were also prepared in pH 7.4 phosphate buffer.

The R_f values of potassium (di)chromate in acidic and alkaline samples are given in Table 2. The presence of neither NaOH nor HCl interferes. The salt concentration due to evaporation also had no effect (3); 0.1–1% NaCl was used as the solvent system for various metals in the previous reports (1–3).

In the chemical reaction, succinate dehydrogenase of pig liver acetone powder or fresh sheep liver acts on the sodium succinate substrate in a dehydrogenation (oxidation) reaction of the

Table 2. Detection of potassium (di)chromate in alkaline and acidic fresh water solutions by paper chromatography

Prepd solns, concd + spotted	Vol. applied, μL	Appearance of paper strip	R_f [a]
Blank 0.01N NaOH	5	no inhibition	—
Blank 0.01N NaOH + buffer	10	no inhibition	—
50 ppm PC[b] in 0.01 NaOH	5	round spot, clear	0.72 ± 0.01
50 ppm PC in 0.01 NaOH + buffer	10	inhibition, compact, clear white chromatogram	0.78 ± 0.02
Blank 0.01N HCl	5	inhibition, compact chromatogram—fast moving at solvent front	1.0 ± 0.1
Blank 0.01N HCl + buffer	10	no inhibition	—
50 ppm PC in 0.01 HCl + buffer	10	inhibition, compact, clear chromatogram	0.73 ± 0.1
Blank 1% NaCl	5	no inhibition	—
50 ppm PC in 1% NaCl + buffer	10	inhibition, compact, clear white chromatogram	0.74 ± 0.03
50 ppm PC in tannery effluent + buffer	10	inhibition, compact, clear white chromatogram	0.75 ± 0.1
Water from 6 abandoned[c] irrigation wells	10	inhibition, compact clear white chromatogram	0.74 ± 0.1

[a] With standard deviation of 6 observations.
[b] Concentration of potassium di(chromate) by 10 times in solutions.
[c] Wells are located in Kara Village near SIPCOT, Ranipet, N.A. Dt., Tamil Nadu State, India.

Krebs cycle to convert the substrate to fumarate (10). The electrons liberated are accepted by tetrazolium salt, INT (11), reducing it to a colored formazan complex. Potassium (di)chromate inhibits the enzyme reaction (11) to produce white inhibition zones in a farmazan background (1-3).

Limitations of the method are that the technique is not suitable for quantitation, and acidity or alkalinity exceeding 0.01N is likely to interfere during detection. The latter problem can be overcome by diluting the alkaline or acidic sample to the recommended strength of 0.01N. The volume of application should not exceed 20 μL for fresh water or 30 μL for acidic or alkaline concentrates. As little as 50 ppm potassium (di)chromate can be detected in fresh water samples subject to concentration by evaporation as described in the method. The detection limit is 250 ppm without concentration. The minimum amount that can be detected by paper strip chromatography is 5 μg; amounts greater than 40 μg cause streaking. Therefore, samples must be concentrated to the detectable range. Presence of potassium (di)chromate can be identified in the acetone-water-EDTA (75 + 20 + 5) solvent system at R_f 0.75. Chromium sulfate shows no mobility and is inhibitory at high concentrations (20 μg). Zinc sulfate, barium chloride and compounds of mercury, copper, cadmium, and silver (1, 2) inhibit on a paper strip or detector strip (3) but are not separated by this solvent system; the recommended solvent system (1, 2) is 0.1% NaCl. The method can be used for fresh water or effluents known to contain potassium (di)chromate (Table 2). In spot testing, the sample is applied on paper and detected without chromatographic separation; 2 μg can be detected by the spot test, thus enhancing sensitivity.

The advantage of the method is that other metal compounds like cobalt, arsenic, aluminum, magnesium, manganese, lead (stannous) chloride, and potassium chloride do not interfere. Potassium (di)chromate can be detected directly in water resources with the least cleanup. For example, 6 irrigation wells of Kara village, abandoned due to underground seepage of potassium chromate from the effluents of a chemical plant, were successfully analyzed for potassium chromate by the spot technique and the micro TLC method.

Acknowledgments

The authors thank R. Ramamurthy, Professor and Head of the Zoology Department, for providing facilities. One author (SRB) thanks CSIR, Government of India, for financial support.

References

(1) Nanda Kumar, N. V., & Prameela Devi, Y. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 729-732
(2) Prameela Devi, Y., & Nanda Kumar, N. V. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 1301-1304
(3) Nanda Kumar, N. V., & Prameela Devi, Y. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 841-843
(4) Maslowask, Z. (1973) *Chem. Anal. (Warsaw)* **18**(2), 389-395

(5) Scott, W. W. (1939) *The Elements*, Vol. 1, 5th Ed., Technical Press Ltd, London, UK
(6) Agterdenbos, J., Van Breckhavers, L., Jute, B. A., & Schuring, J. (1972) *Talanta* **19**, 341–345
(7) Saltzman, B. E. (1952) *Anal. Chem.* **24**, 1016–1019
(8) Parker, C. R. (1976) "Water Analysis by Atomic Absorption Spectroscopy," Varian Techtron Manual, Walnut Creek, CA, pp. 46–47
(9) Bergmeyer, H. V. (1965) *Methods of Enzymatic Analysis*, Academic Press, New York, NY
(10) Meyes, P. M. (1969) *Review of Physiological Chemistry*, 12th Ed., Medical Publication, Maruzen Co., Asian Edition, Japan, pp. 172–193
(11) Seligman, A. M., Kwan-Chung-Tsow, X., Choashing Chang-in-Mann, N. N. (1956) *J. Am. Chem. Soc.* **78**, 6139–6144

Microdetermination of Ethylene Dibromide in Air by Gas Chromatography

T. DUMAS and E. J. BOND
Agriculture Canada, Research Centre, University Sub Post Office, London, Ontario, Canada N6A 5B7

Using a new, sensitive photo-ionization detector in a portable gas chromatograph that operates at ambient temperature, a method was developed for determining ethylene dibromide in air as low as 19 ppb. Vapor standards were prepared for calibration of the detector; concentrations in these standards were accurately determined by using a flame ionization detector. This detector was previously calibrated with an aqueous standard of ethylene dibromide. Using the 2 gas chromatographs, the loss of ethylene dibromide through sorption from the atmosphere of the vapor standards was determined. Solubility of ethylene dibromide in water was also established at 3 temperatures, using the flame ionization detector.

The extensive use of ethylene dibromide, a possible human carcinogen, as a gasoline additive and as an agricultural fumigant has brought about the need for reliable, precise determination of this vapor in the atmosphere. In 1980, the American Conference of Governmental Industrial Hygienists (1) listed ethylene dibromide as a substance associated with industrial processes and recognized to have carcinogenic potential, but it was not assigned a threshold limit value. A level as low as 0.015 ppm has been proposed by some agencies as a threshold limit, but methods for determining very low levels under field conditions have not been available. Furthermore, methods for establishing reliable vapor standards of a highly sorptive compound like ethylene dibromide needed investigation for accurate calibration of gas chromatography detectors.

A sensitive and portable gas chromatograph that will analyze both low and high levels of fumigants like ethylene dibromide has recently become available (2, 3). The present publication reports the results of tests with the portable gas chromatograph to determine low levels of ethylene dibromide vapor and describes methods for developing standards for these tests.

Experimental

To determine EDB vapor concentration, it was first necessary to produce aqueous standards for calibration of a gas chromatograph with a flame ionization detector (FID) so that vapor standards of known concentration could be prepared. Vapor standards were required because the Photovac 10A10 gas chromatograph (with a photo-ionization detector (PID)) operates at ambient temperature where aqueous samples can not be analyzed.

Aqueous standards were prepared by mixing 10 μL ^{14}C-ethylene dibromide (0.0005 mC/mL, previously prepared by diluting 1 μL of 9.35 mC/mmol with 1 mL nonlabeled ethylene dibromide) with 100 mL water. Activity of the standards was determined by injecting 10 μL ethylene dibromide solution in 15 mL fluor (800 mL ethylene glycol monomethyl ether, 1 L toluene, 4 g omnifluor) and counting in a Beckman L S 9000 scintillation counter. Aqueous standards were compared with standards prepared in methanol, in which ethylene dibromide is known to be soluble. Methanol standards could not be used in the gas chromatograph because of excessive response of the detector to the solvent.

To ascertain that all ethylene dibromide could be dissolved in water at the concentration used, saturated aqueous solutions were prepared by storing excess ethylene dibromide with water for several days at several temperatures and then determining the concentration in the aqueous phase.

Using these aqueous standards, a flame ionization detector in a Bendix Model 2300 gas chromatograph was calibrated so that the precise concentration of vapor standards for use in the Photovac 10A10 gas chromatograph could be determined. Using a 2 m × 3 mm id stainless steel column packed with 5% Carbowax 20M on 60–80 mesh Chromosorb W, 30 mL/min nitrogen flow rate, and 120°C column temperature, a 2.3 min retention time was obtained on the Bendix gas chromatograph. Sensitivity was 2 μg EDB for full scale chart deflection. Data were calculated with a Hewlett-Packard 3380 integrator.

Standards of EDB in air were prepared by injecting 0.2–100 μL liquid aliquots into 12.6 L flasks. Reagent grade liquid EDB was vaporized

Received February 11, 1982. Accepted April 14, 1982.

0004-5756/82/6506-1379–03$01.00

Table 1. Activity of labeled ethylene dibromide dissolved in methanol or water

Sample[a]	Counts per min	
	Methanol	Water
1	757.43	713.84
2	732.23	677.89
3	720.24	718.52

[a] 10 μL sample in 100 mL solvent.

Table 2. Solubility of ethylene dibromide in water at various temperatures

Temp., °C	Satn level, μg/μL	EDB in aq. soln	
		Applied, μg/μL	Found, μg/μL
10	2.0	—	—
25	3.4	0.217[a]	0.23
25	3.4	1.08[b]	1.17
31	4.3	—	—

[a] 10 μL EDB in 100 mL water.
[b] 50 μL EDB in 100 mL water.

in the flask by spot heating with a flame. The EDB content of these standards was determined with the Bendix 2300 gas chromatograph equipped with a flame ionization detector. Then, the Photovac 10A10 portable gas chromatograph with a photo-ionization detector was calibrated with these known vapor standards on a column of 2 m × 3 mm id Teflon TFE packed with 80–100 mesh Superpack 20M. At a temperature of 25°C and 10 mL/min flow of high purity (less than 0.1 ppm hydrocarbons) air carrier gas, a 7 min retention time was obtained.

Results and Discussion

The results in Table 1 show that ethylene dibromide dissolved in water to a similar extent as in methanol, and therefore aqueous standards were deemed suitable for the flame ionization detector.

The extent of ethylene dibromide solubility in water is shown by the data in Table 2. At 3 temperatures, 31, 25, and 10°C, the equilibrium saturation amounts were 4.3, 3.4, and 2.0 μg/μL. When 10 and 50 μL ethylene dibromide were dissolved in 100 mL water, 0.23 and 1.17 μg/μL were found; this compared closely with calculated amounts of 0.217 and 1.08 μg/μL, and indicated that all the ethylene dibromide was dissolved in the water.

Results of analysis of ethylene dibromide in the vapor phase by the flame ionization and photo-ionization detectors are given in Table 3. With a 1 mL sample containing 0.00015 μg ethylene dibromide, the PID gave 25% full scale response on the recorder. This represents a concentration of 19 ppb and indicates that the detector could be used for determining concentrations somewhat below this level.

In comparison with the FID, which gave relatively accurate data as low as 0.55 μg/mL, the PID was sensitive at levels as low as 0.00015 μg/mL.

The results in Table 3 also show the sorption of ethylene dibromide by the glass surface of the containers at different concentrations. At the low dosage where 0.0003 mg/L was applied, the amount of EDB remaining in the atmosphere after 2 days was one-half the amount applied; at the higher dosages of 1.72 and 17.2 mg/L, it had decreased to about one-third. Beyond the 2-day period, the decline in concentration was very slow so that the atmosphere in the flask was relatively constant and satisfactory for use as a standard.

All these results show that the Photovac 10A10 gas chromatograph with a photo-ionization detector can determine ethylene dibromide over a

Table 3. - Concentration of ethylene dibromide, as determined by photo-ionization (PID) and flame ionization (FID) detectors, when vaporized in 6 L desiccator or 12.6 L flasks and analyzed over a period of 48 h

Detd after, h	Concentration of EDB, mg/L[a]				
	0.0003[b]	0.034[c]	1.72[c]	1.72[c]	17.2[c]
	PID	PID	PID	FID	FID
1	0.00022	0.022	—	1.6	19
2	0.00023	0.025	—	1.11	8.8
4	0.0002	0.019	—	1.0	10.9
6	0.0002	0.018	0.88	0.95	10.9
24	0.00017	0.016	0.76	0.87	7.5
48	0.00015	0.013	0.76	0.55	6.5

[a] All results are averages of 2 determinations.
[b] EDB applied in 6 L desiccator.
[c] EDB applied in 12.6 L flasks.

wide range of concentrations to a level as low as 19 ppb. The higher concentrations can be determined by reducing sample size and increasing attenuation of the signal.

REFERENCES

(1) American Conference of Governmental Industrial Hygienists (1980) "Threshold limit values for chemical substances and physical agents in the working environment," Cincinnati, OH

(2) Barker, N. J., & Leveson, R. C. (1980) *Am. Lab.* Dec., 76–83

(3) Bond, E. J., & Dumas, T. (1982) *J. Agric. Food Chem.*, in press

Gas Chromatographic Determination of the Captan Metabolite Tetrahydrophthalimide in Urine

SARAH R. SCHOEN and WRAY L. WINTERLIN
University of California, Environmental Toxicology Department, Davis, CA 95616

A gas-liquid chromatographic (GLC) method is described for the determination of the captan metabolite tetrahydrophthalimide in human urine. The sample is extracted with chloroform and cleaned up on a Florisil column. The extract is then analyzed by GLC, using a nitrogen-phosphorus-selective detector. Recoveries from urine samples fortified at 0.03–0.5 ppm ranged from 82 to 87%, and were qualitatively confirmed by GLC-mass spectrometry.

Captan (*N*-trichloromethyl-4-cyclohexene-1,2-dicarboximide) has been used as a fungicide for over 25 years on a number of fruit and vegetable crops. Recently, because of reported potentially toxic effects, the Environmental Protection Agency placed captan on its list for Rebuttable Presumption Against Registration (RPAR).

As far as human exposure is concerned, the highest risk is to agricultural workers employed in the fields during and after spraying, and to pesticide applicators, mixers, and loaders. Because no preharvest interval for worker re-entry has been established, the levels of exposure could be quite high. How this exposure relates to worker safety is not known. Therefore, it is essential to have a biological index for measuring captan exposure. Because a study with radiolabeled metabolites showed tetrahydrophthalimide (THPI) to be a major metabolite of captan orally administered to rats (1), THPI was chosen as a biological index of captan exposure. Until now no analytical procedure for measuring THPI in biological fluids, such as urine, has been published.

This paper describes a gas-liquid chromatographic (GLC) procedure to determine levels of THPI in human urine, which could be used as an indicator of worker exposure to captan.

METHOD

Reagents

(a) *Solvents.*—Chloroform, nanograde (Mallinckrodt Chemical Works, St. Louis, MO 63160); benzene and acetone, Resi-analyzed (J.T. Baker Chemical Co., Phillipsburg, NJ 08865); ethyl ether, anhydrous, AR grade (Mallinckrodt Chemical Works); ethyl acetate, double-distilled, AR grade (Mallinckrodt Chemical Works).

(b) *Florisil.*—As received, PR grade, 60–100 mesh (Floridin Co., Berkeley Springs, WV 25411).

(c) *Sodium sulfate.*—Anhydrous, AR grade (Mallinckrodt Chemical Works).

(d) *THPI standard.*—mp 134–138°C (Aldrich Chemical Co., Inc., Milwaukee, WI, 53233). Prepare stock 1 mg/mL solution by adding 100 mg THPI to 100 mL volumetric flask and diluting to volume with double-distilled ethyl acetate. Make dilutions as necessary for sample fortification and standard curves.

Apparatus

(a) *Chromatographic column.*—12 × 10 mm, 200 mL reservoir.

(b) *Rotary evaporator.*—CaLab Model C (California Laboratory Equipment Co., Berkeley, CA).

(c) *Nitrogen evaporator.*—N-Evap Model 106, (Organomation Associates, Inc., Shrewsbury, MA 01545).

(d) *Sedimentation tubes.*—6.5 mL capacity, Kimax (Owens-Illinois, Inc., Vineland, NJ 08360).

(e) *Gas chromatograph*—Hewlett-Packard Model C 5701 equipped with nitrogen-phosphorus detector, Model 18789A. Operating parameters: 180 cm × 2 mm id coiled glass column, first 130 cm packed with 4.5% OV-225, second 50 cm packed with 4% OV-101, both on modified 80–100 mesh Gas-Chrom Q; column 200°C, injection port 200°C, detector 300°C; helium carrier gas 30 mL/min, air 60 mL/min, hydrogen 4 mL/min; Honeywell Electronik 16 recorder, chart speed 4 min/in.; THPI retention time 2.1 min.

(f) *Mass spectrometer.*—Finnigan 4021 with INCOS 2100 Data System. Operating parameters: 1.0 m × 2 mm id glass column packed with 3% OV-225 on 80–100 mesh Gas-Chrom Q; column temperature programmed from 200 to 240°C, source 280°C, manifold 100°C; electron impact at 70 eV; THPI retention time 1.3 min.

Extraction

Volumetrically pipet 25 mL urine into 250 mL separatory funnel containing 50 mL chloroform.

Received November 25, 1981. Accepted April 4, 1982.

0004-5756/82/6506-1382-03$01.00

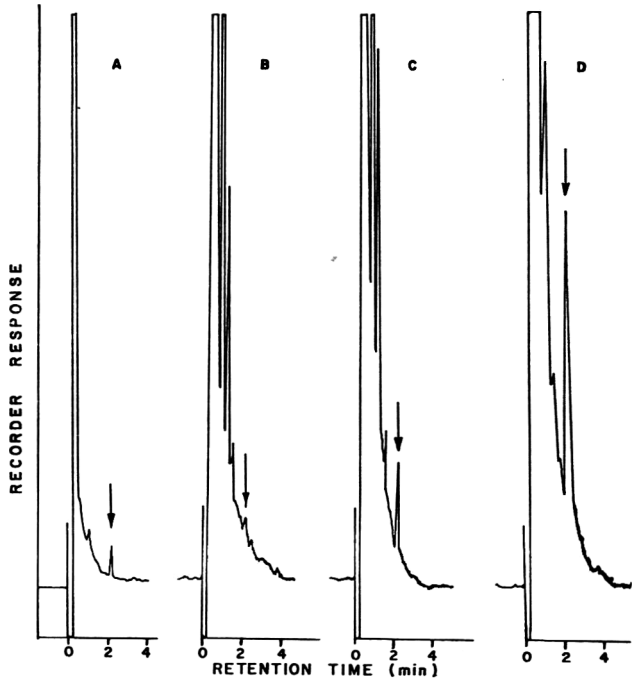


Figure 1. Tracings of typical samples, using OV-225/OV-101 column and nitrogen-phosphorus detector: A, 1 ng injection of THPI standard in 4 μL ethyl acetate; B, 100 mg control urine injection in 4 μL ethyl acetate; C, 100 mg control urine fortified at 0.03 ppm THPI; D, 33.3 mg field worker urine sample in 4 μL ethyl acetate (0.28 ppm THPI).

Shake gently 30 s and let layers separate. Filter chloroform layer through glass funnel plugged with glass wool containing 100 g anhydrous sodium sulfate. Collect in 250 mL round-bottom flask. Re-extract sample 2 more times with 50 mL chloroform and collect extracts as in previous step. Wash sodium sulfate contained in funnel with 25 mL chloroform and rotary-evaporate sample extract to near-dryness at 40°C. Add 5 mL benzene and re-evaporate to near dryness. Dissolve residue with 1 mL benzene in preparation for Florisil column cleanup.

Column Cleanup

Prepare chromatographic column as follows: Insert glass wool plug followed by 0.5 cm anhydrous sodium sulfate, 9.5 mL Florisil, and top with a final 0.5 cm anhydrous sodium sulfate. Wet column with 25 mL benzene. When benzene is just above top of column, quantitatively transfer sample extract with disposable pipet. Rinse flask 3 times with total volume of 10 mL benzene and transfer rinses to top of column. Rinse flask with additional 10 mL benzene and transfer rinse to top of column. Wash column with 125 mL 5% ethyl ether in benzene and discard wash. Elute THPI with 75 mL 8% acetone in benzene and collect in 250 mL round-bottom flask. Rotary-evaporate just to dryness. Add 5 mL ethyl acetate and re-evaporate to near dryness. Quantitatively transfer extract to sedimentation tube, using a total of 6 mL ethyl acetate. Concentrate on nitrogen evaporator to no less than 1 mL. Final adjusted volume will depend on residue levels in cleaned up extracts. However, for minimum detectable levels, up to 4 μL from concentrate of 1 mL may be injected onto gas chromatograph.

Recovery Study

Using 25 μL Hamilton syringe, samples were fortified at 0.03 ppm by letting 7.5 μL of a solution containing 100 ng THPI/μL ethyl acetate completely air-dry; 25 mL urine was added and shaken thoroughly before chloroform extraction was begun. Fortifications at 0.05 ppm and 0.50 ppm were carried out using 12.5 μL of solutions containing 100 ng THPI/μL ethyl acetate and 12.5 μL of solutions containing 1.0 μg THPI/μL ethyl acetate, respectively.

Results and Discussion

The only previously published method for THPI concerns analysis of milk and meat (2).

The procedure used electron capture GLC following derivatization. By using a gas chromatograph with a nitrogen-phosphorus detector instead of electron capture, the method presented in this paper requires only one cleanup column instead of two, avoids the derivatization step, and is more specific for nitrogen-containing compounds. Recoveries and sensitivity of the 2 methods, however, are similar. Figure 1, A–D, shows representative chromatograms of standard, recovery, and field samples injected onto the gas chromatograph containing a nitrogen-phosphorus detector. Figure 1A is a chromatogram representing a 1 ng injection of THPI standard which was the minimum quantity used for detection. For 25 mL samples concentrated to 1 mL ethyl acetate and using 4 μL injections, this represents a detection limit of 0.01 ppm.

The major difficulty in the urine analysis was the presence of an interfering response in the control samples (Figure 1B), which was generally less than 0.01 ppm. Urine samples from 5 men and 5 women who had not been exposed to captan showed one sample equivalent to 0.01 ppm and another, 0.015 ppm THPI, both from males. Although a variety of different gas chromatographic columns were tried, we were unsuccessful in resolving this peak from THPI. An effort to identify this peak by GLC/MS was also unsuccessful because of the limit in sensitivity of the GLC/MS system. Because of this interference, our minimum detectable level for the urine samples was therefore set at 0.03 ppm. Figure 1C depicts the same control sample as Figure 1B, fortified at 0.03 ppm.

Recoveries of THPI are as follows: At fortification levels of 0.03, 0.05, and 0.50 ppm THPI, the recoveries were 82, 87, and 86%, respectively. The respective standard deviations were 10.5, 5.2, and 5.8%. These recoveries represented an average of 9 samples at 0.03 ppm THPI and 5 samples each at 0.05 and 0.50 ppm THPI.

Using the method as described here, approximately 12 urine samples per 8 h day can be analyzed. Although this study was restricted to urine taken from humans, the method should be adaptable to urine analysis for metabolism studies in other animals.

Figure 1D depicts a sample taken from a farmworker exposed to captan in the field; the 0.28 ppm THPI found was qualitatively confirmed by GLC/MS. Detailed results of a farmworker study involving exposure to captan will be published by Winterlin et al. in a subsequent paper.

Acknowledgments

The authors thank John C. McKay of Stauffer Chemical Co., Richmond, CA, for supplying valuable background information, and Benjamin Giang, also of Stauffer, for mass spectrometry.

References

(1) Environmental Protection Agency/Office of Pesticide Programs. Criteria and Evaluation Division, Substitute Chemical Program: Initial Scientific and Minieconomic Review of Captan, EPA-540/1-75-012 (1975), pp. 58–59

(2) Onley, J. H. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 679–681

Spectrophotometric Determination of Oxidizing Substances in Food-Grade Hydrochloric Acid

BRIAN J. LEVEY and PAUL F. REIGLER
The Dow Chemical Co., Analytical Laboratories, Midland, MI 48640

A spectrophotometric method has been developed for the determination of oxidizing substances in food-grade hydrochloric acid. The method is based on the reaction of oxidizing substances with potassium iodide to form triiodide ion (I_3^-) whose absorbance is then measured at 485 nm. Oxidizing substances is reported as μg Cl_2/mL. The method is applicable to oxidizing substances in the range of approximately 9-37 μg Cl_2/mL.

Oxidizing substances (as Cl_2) is a Food Chemicals Codex (FCC) specification for hydrochloric acid (HCl). The FCC designated method is a limits test (not more than 30 ppm) (1).

In order to routinely monitor the level of oxidizing substances in HCl, a simple, rapid method is needed, which provides a defined value for oxidizing substances rather than a limit test. Several different techniques were investigated for applicability to this problem, including potentiometric, titrimetric, colorimetric, and ultraviolet spectrophotometric methods (2-5). The spectrophotometric method described in this paper was selected because it provides good accuracy and precision, and is ideally suited for use in a quality control laboratory. The method is based on spectrophotometric measurement of triiodide ion produced by the reaction of potassium iodide with oxidizing substances.

METHOD

Apparatus

(a) *Spectrophotometer.*—Varian DMS 90 UV/VIS, with 1.0 cm glass cells.

(b) *Polyethylene bottles.*—1 oz, with Polyseal® caps.

Reagents

(a) *Hydrochloric acid.*—36.5-38%, ACS, reagent grade (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(b) *Potassium iodide.*—Crystals, reagent grade (J. T. Baker Chemical Co.).

(c) *Potassium iodide solution.*—10%. Dissolve 10 g potassium iodide in 90 mL deionized water.

(d) *Potassium iodate.*—Crystals, primary standard (J. T. Baker Chemical Co.).

Received June 18, 1981. Accepted April 14, 1982.

(e) *Iodate stock solution.*—1 mL = 1000 μg Cl_2. Weigh 1.00 g dried potassium iodate into 1 L volumetric flask, dilute to volume with deionized water, and mix thoroughly.

Preparation of Standard Curve

In a series of five 1 oz polyethylene bottles equipped with Polyseal caps, add, by pipet, 0, 0.25, 0.50, 0.75, and 1.00 mL portions of iodate stock solution and, correspondingly, 1.00, 0.75, 0.50, and 0.25, and 0 mL deionized water. By pipet, add 25.0 mL HCl to each bottle. Then add 1.0 mL 10% KI solution to each bottle, quickly cap, and shake each bottle a few times. Let solutions stand 30 min at room temperature.

Adjust spectrophotometer to read zero absorbance at 485 nm on deionized water in 1.0 cm cell using air as reference. After development, read absorbance of each standard solution and record value to 0.001 absorbance unit. Subtract absorbance of blank from each absorbance reading for standard solutions. Plot adjusted absorbance values vs μg Cl_2/27 mL on rectangular coordinate paper. Standard curve obtained is linear in working range of method and was very reproducible.

Determination

By pipet, add 25.0 mL food-grade HCl to clean, dry 1 oz polyethylene bottle, and then add 1.0 mL deionized water and 1.0 mL 10% KI solution. Cap bottle, shake it a few times, and let solution stand 30 min at room temperature.

With spectrophotometer adjusted to read zero absorbance at 485 nm on deionized water in 1.0 cm cell against air as reference, measure absorbance of sample solution. Convert absorbance measurement to μg Cl_2 by using standard curve. One assumption made is that any color observed in sample solution is due to liberated triiodide ion. If untreated HCl sample is colored, run sample blank to account for this reference background.

Discussion

No absorbance maximum was observed for I_3^- in HCl. A wavelength of 485 nm was selected for the experimental work because sensitivity is optimized in this region.

0004-5756/82/6506-1385-03$01.00

A 30-min development period was chosen to ensure complete reaction of low levels of oxidizing substances with iodide. Under the conditions of the method, the relationship between concentration and color intensity obeyed Beer's Law in the range specified ($r^2 = 0.999$).

In the early stages of developing the present method, we used a sodium hypochlorite (NaOCl) stock solution to prepare the calibration curve. This stock solution was standardized by sodium thiosulfate titration, buffered at pH 4 with glacial acetic acid, and calculated as μg Cl_2/mL. A method based on using this stock solution was developed and reviewed internally. It was pointed out that sodium chlorate ($NaClO_3$) might be present in the stock solution and that it might not be accounted for in the standardization procedure, but would, however, readily liberate I_3^- in a concentrated HCl matrix (V. A. Stenger and L. R. Swim, The Dow Chemical Co., Midland, MI). Therefore, we investigated the influence of the $NaClO_3$ in the NaOCl stock solution and its effect on the method.

A NaOCl stock solution was prepared by diluting 50.0 mL NaOCl solution (Baker Analyzed Reagent, 5%) to 1 L with deionized water. This solution was standardized by thiosulfate titration at pH 4 and contained 1860 μg Cl_2/mL. Ion chromatographic determination of sodium chlorate in the same solution showed a concentration of 420 μg $NaClO_3$/mL (V. T. Turkelson, The Dow Chemical Co., Midland, MI). With the presence of $NaClO_3$ substantiated, we designed an experiment to determine at what acid concentration $NaClO_3$ would be accounted for in a thiosulfate titration. Twenty-five mL aliquots of the aqueous NaOCl standard were pipetted into 250 mL Erlenmeyer flasks and then diluted with various volumes of concentrated HCl and deionized water to yield water-to-acid dilution ratios ranging from 8:1 to 1:1. The total volume in each case was maintained at 85 mL. Each sample was prepared immediately before analysis, followed by the addition of approximately 1 g potassium iodide crystals. The solution was swirled briefly (about 5 s), and then titrated immediately with 0.10N $Na_2S_2O_3$. Starch solution indicator was added when a straw-yellow color was attained and the titration was continued until the blue color disappeared.

Volumes of 13.3, 13.3, 13.6, 15.4, and 21.3 mL 0.10N HCl were required to titrate solutions having water-to-acid ratios of 8.0:1, 7.5:1, 3.3:1, 1.8:1, and 1.1:1, respectively. The standardization procedure carried out at pH 4 required 13.3 mL 0.10N HCl. It is apparent that a water-to-acid dilution ratio of at least 1:1 is required before chlorate will readily liberate I_3^-. Thiosulfate titration standardization of NaOCl at pH 4 does not account for chlorate that is present in solution. A standard chlorine curve constructed on the basis of this standardization would include a significant positive interference from the chlorate, resulting in values for oxidizing substances that have a significant negative bias. In order to use NaOCl as a standard in this method, the standardization procedure would have to account for the $NaClO_3$ present in solution. This was accomplished by performing the sodium thiosulfate titrations under strongly acidic conditions. The results obtained by this procedure, however, were very erratic.

For these reasons, potassium iodate was selected for use in the development of the present method and, in general, this represents a better alternative (5, 6). Potassium iodate is a primary standard, which liberates a stoichiometric quantity of triiodide ion under the conditions of the method, forms a very stable solution, and experiences no variation in titrant volumes when a known quantity is titrated with sodium thiosulfate at varying water-to-acid dilution ratios.

The spectrophotometric method described, utilizing a potassium iodate stock solution, is not specific. Any substance capable of oxidizing iodide to I_3^- under the conditions of the method will contribute.

The method is sensitive to temperature; however, small variations in temperature (±5°C) will not significantly affect the accuracy of the method (Table 1).

In the ASTM method, free chlorine quantitatively bleaches methyl orange in strongly acidic solution and is determined spectrophotometrically at 505 nm after a 30-min development period (W.E. LeRoux, Hooker Chemical Co., Niagara Falls, NY; ASTM Subcommittee E-15.51, Mineral Acids). The method specifies the preparation of a standard chlorine solution of approximately 10 ppm by serial dilution of household bleach (NaOCl). This stock solution is standardized against 0.01N sodium thiosulfate in a titration buffered at pH 4 with glacial acetic acid. The contribution of $NaClO_3$ on the oxidation of dilute methyl orange solution in a

Table 1. Effect of temperature on results obtained for oxidizing substances (μg Cl_2/mL) in food-grade HCl

Sample	15°C	25°C	35°C
A	4.9	5.3	6.3
B	15.3	16.0	17.0

Table 2. Precision of spectrophotometric method for determining oxidizing substances (μg Cl_2/mL) in food-grade HCl

Trial	Sample 1	Sample 2
1	22.3	7.2
2	21.5	7.9
3	22.1	7.2
4	24.0	7.2
5	22.6	7.8
6	23.5	7.3
7	24.4	7.2
8	23.2	8.4
9	21.8	7.0
10	23.2	9.3
Mean	22.8	7.7
SD	0.96	0.72
RSD, %	4.2	9.4

Table 3. Recovery study for oxidizing substances in food-grade HCl [a]

	μg Cl_2/27 mL		
Sample	Added	Found	Rec., %
1	250	241	96.4
	500	471	94.2
	750	754	100.5
2	100	99	99.0
	200	204	102.0
	400	422	105.4

[a] Each value is the average of 2 separate determinations.

strongly acidic medium has not been accounted for because $NaClO_3$ is not accounted for in the standardization procedure.

Results

The precision of the method was determined by analyzing 5 portions of 2 different samples on 2 consecutive days. The relative standard deviations at the 8 and 23 μg Cl_2/mL levels were 9.4 and 4.2%, respectively (Table 2).

Recovery was measured by adding known amounts of the iodate stock solution to 25 mL portions of the 2 different food-grade HCl samples used in the precision study and carrying out the determination. An average recovery of 99.7% was determined for the concentration ranged specified in the method (Table 3).

References

(1) *Food Chemicals Codex* (1972) Second Ed., National Academy of Sciences, Washington, DC, p. 375
(2) "Annual Book of ASTM Standards" (1976) Standard D-1253-76, American Society for Testing and Materials, Philadelphia, PA, p. 276–282
(3) Brown, K. D., & Parker, G. A. (1979) *Anal. Chem.* **51**, 1332–1333
(4) Brooks, A. S., & Seegert, G. L. (1979) *J. Water Pollut. Control Fed.* 2636–2640
(5) Jenkins, R. L., & Baird, R. B. (1979) *Anal. Lett.* **12**(A2), 125–141
(6) Ringdon, L.P., Moody, G. J., & Frazer, J. W. (1978) *Anal. Chem.* **50**, 465–468

FOOD ADDITIVES

Gas Chromatographic and Gas Chromatographic-Mass Spectrometric Confirmation of 2,4- and 2,6-Toluenediamine Determined by Liquid Chromatography in Aqueous Extracts

ROGER C. SNYDER, WILLIAM C. BRUMLEY, CHARLES V. BREDER, and THOMAS FAZIO

Food and Drug Administration, Division of Chemistry and Physics, Washington, DC 20204

The confirmation of 2,4- and 2,6-toluenediamine (TDA) in aqueous extracts from boil-in-bags and retortable pouches is described. The extracts were initially analyzed by a high performance liquid chromatographic procedure and any apparent 2,4- and/or 2,6-TDA were quantitated. The liquid chromatographic effluent corresponding to any apparent 2,4- or 2,6-TDA was collected. TDA was then partitioned into ethyl acetate and reacted with trifluoroacetic anhydride (TFAA). The TDA-TFAA derivative formed was confirmed by gas-liquid chromatography (GLC) using a 1.2 m × 0.32 cm nickel column packed with 6% OV-17 on Superpak-20M. Results obtained from analyzing extracts of several retortable pouches and boil-in-bags showed levels of TDA migration ranging from <0.1 to 2.2 ppb (μg/L). Additional confirmation of the TDA-TFAA derivative from retortable pouches by multiple ion detection GC/mass spectrometry is also described.

Numerous components of polymeric food packaging materials have the potential to migrate from the polymer matrix into the food. Toluenediisocyanate (TDI), a component of certain adhesives used to laminate boilable and retortable plastic food pouches, is an example of such a potential migrant. Commercial TDI generally is produced as an isomeric mixture consisting primarily of 2,6-TDI (about 20%) and 2,4-TDI (about 80%) (1). TDI is readily hydrolyzed to toluenediamine (TDA) and, as a result, any migrating TDI would appear as the diamine. This potential migration of TDI is of concern because animal feeding studies have indicated that 2,4-TDA is carcinogenic (2, 3).

This laboratory previously developed a high performance liquid chromatographic (HPLC) procedure capable of measuring ≥50 ppt (ng/L) of apparent 2,4- and 2,6-TDA in aqueous extracts. Recovery and precision for this method were 89 ± 4% (4). Unless verified by an identification technique, however, all TDA observed by the HPLC procedure must be considered as apparent TDA. Therefore, a gas-liquid chromatographic (GLC) procedure for confirming the identity of apparent TDA measured by the HPLC procedure was developed and is reported here. In addition, the identity of several samples was confirmed by multiple ion GLC/mass spectrometry (MS) and this technique is also presented.

Received January 26, 1982. Accepted April 15, 1982.

METHOD

Apparatus

(a) *Flash evaporator.*—Buchler Instruments, Fort Lee, NJ 07024.

(b) *Mini-vials.*—3 mL tapered with screw caps and Teflon-lined septa (Alltech Associates, Deerfield, IL 60015).

(c) *Heat sealer.*—Sentinel Model 12AS (Packaging Industries, Hyannis, MA). Sealed at 191°C under 15 psi clamp pressure for 2 s.

(d) *Liquid chromatograph.*—DuPont Model 850 (DuPont Instruments, Wilmington, DE 19898) equipped with fixed wavelength ultraviolet (UV) detector, column oven compartment, and Model 7125 Rheodyne injector valve (Rheodyne Inc., Cotati, CA 94928). The UV detector was connected in series to Spectrum Model 1021A electronic filter/amplifier (Spectrum Scientific, Newark, DE 19711), operated at frequency cutoff of 0.02 Hz, and Hewlett-Packard 7130A strip chart recorder (Hewlett-Packard Co., Palo Alto, CA 94304). Operating conditions: flow rate 1 mL/min; oven temperature 40°C; UV detector 254 nm, 0.002 absorbance unit full-scale deflection; injection volume 200 or 500 μL. Mobile phase 16% methanol–84% aqueous phosphate buffer consisting of 0.001M NaH_2PO_4–0.003M Na_2HPO_4, pH 7.4.

(e) *LC columns.*—For retorted pouch extracts, 4.6 × 250 mm column packed with 5 μm Ultrasphere-octyl (Altex Scientific, Inc., Berkeley, CA 94710). For boil-in-bag extracts, 4.6 × 150 mm column packed with 3 μm Spherisorb-ODS-2 (Chromanetics Corp., Baltimore, MD 21236).

(f) *Gas chromatograph.*—Hewlett-Packard

0004-5756/82/6506-1388-07$01.00

5840A equipped with N/P detector. Operating conditions: temperatures (°C)—oven 200, injection port 210, detector 210; nitrogen carrier gas 20 mL/min; hydrogen 5.5 mL/min; air 40 mL/min; N/P detector 16–20 V. Injection volume 5 μL.

(g) *GLC column.*—1.2 m × 0.32 cm nickel packed with 6% OV-17 on Superpak-20M.

(h) *Gas chromatograph/mass spectrometer.*—Finnigan A Model 3300F electron ionization quadrupole mass spectrometer with Model 9500 gas chromatograph and Finnigan INCOS 2300 data system (Finnigan Corp., Sunnyvale, CA 94086). Multiple ion detection (MID) of ions at m/z 104, 148, 243, 245, and 314. Operating conditions: temperatures (°C)—injector 230, glass jet separator 260, transfer line 230; helium carrier gas 25 mL/min. Column: same as (g), column temperature 210°C; solvent divert time 3 min (data acquisition started at 3.25 min). Mass spectrometer parameters: pre-amp and multiplier 10^{-8} V/A, 2200 V; electron energy 70 eV; emission current 0.50 mA. Retention times of 2,6- and 2,4-TDA were ca 3.6 and 4.0 min, respectively.

Reagents and Materials

(a) *2,4- and 2,6-TDA.*—98% pure (Aldrich Chemical, Milwaukee, WI 53233).

(b) *Trifluoroacetic anhydride (TFAA).*—Reagent grade (Fisher Scientific, Pittsburgh, PA 15219).

(c) *Methylene chloride and ethyl acetate.*—Distilled-in-glass, pesticide grade (Burdick & Jackson, Muskegan, MI 49445).

(d) *Methanol.*—HPLC grade (Fisher Scientific).

(e) *Water.*—Prepared by passing house distilled water through Milli-Q (Millipore Corp., Bedford, MA 01730) water purification system.

(f) *Mono- and disodium phosphate buffers.*—Certified ACS grade (Fisher Scientific).

(g) *Retortable pouches.*—Supplied by 4 manufacturers.

(h) *Boil-in-bags.*—Five varieties purchased locally.

Procedure

All 4 retortable pouches and 2 of the 5 boil-in-bag samples were used as received. The other boil-in-bag samples were purchased containing frozen food, which was removed, and bags were rinsed clean before testing.

Aqueous extracts.—The boil-in-bag aqueous extracts were obtained by filling the bags with Milli-Q purified water (1 mL/2.6 sq. cm), heat-sealing, immersing in a boiling water bath (ca 100°C) for 2 h, and transferring the contents while hot to suitable glass storage containers. Retorted pouch aqueous extracts were similarly obtained by filling the pouches with Milli-Q water (1 mL/1.6 sq. cm), heat-sealing, and retorting for 2 h (121°C, 15 psi). The pouches were allowed to cool and were stored unopened at ambient temperature until analyzed.

Sample cleanup and concentration.—Before LC, the aqueous sample extracts were washed and concentrated by the following methylene chloride extraction procedure: 250 mL sample extract was transferred to a 500 mL separatory funnel, 1 mL 0.4M HCl was added, and the solution was then shaken with 100 mL methylene chloride. The methylene chloride layer, which contained the acidic and neutral extractable components, was drained and discarded. The aqueous layer was made basic by the addition of 1.5 mL 0.4M NaOH, followed by extraction with two 150 mL portions of methylene chloride. The methylene chloride extracts, which totaled 300 mL, were combined in a 500 mL round-bottom flask. Four mL 0.01M HCl was added as a holding solvent and the methylene chloride was removed under vacuum (ca 5 mm Hg) at 30°C. The 4 mL sample concentrate was transferred to a 5 mL volumetric flask, using a disposable pipet, and neutralized by diluting to volume with a solution of 0.04M NaOH, 0.005M NaH_2PO_4, and 0.015M Na_2HPO_4. The final 5 mL volume represented a 50-fold concentration of the original aqueous extract.

LC and fractionation.—For retorted pouch extracts 500 μL and for boil-in-bag extracts 200 μL of the 50-fold methylene chloride concentrate was injected into the liquid chromatograph. Apparent 2,4-and/or 2,6-TDA were quantitated by the external standard technique.

The HPLC conditions are given under *Apparatus.* Either of the 2 LC columns described can be used; both yielded similar chromatograms of TDA. The 3 μm Spherisorb-ODS-2 column, being only 4.6 × 150 mm, gave shorter retention times for TDA; however, it also required a smaller injection volume (200 μL).

For subsequent GLC, fractions of the LC effluent corresponding to the 2,4- and 2,6-TDA elution volumes were collected in 3 mL minivials. Two 1 mL fractions were collected for the retorted pouch extracts and two 300 μL fractions for the boil-in-bag extracts. For the boil-in-bag extracts, duplicate LC injections were made and the collected fractions were combined to give adequate GLC sensitivity.

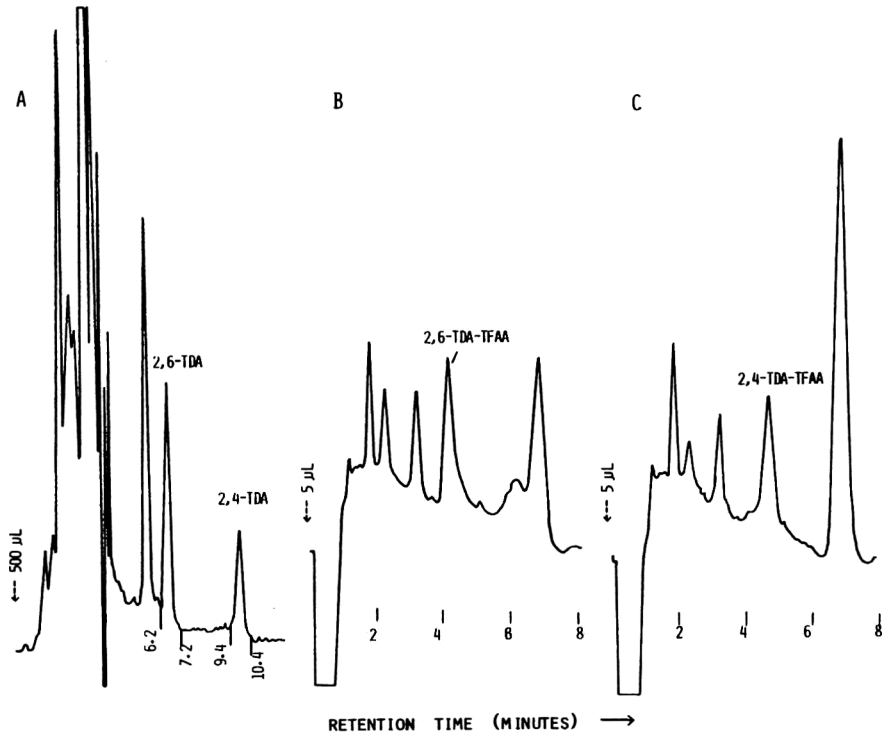


Figure 1. A, 500 µL LC injection of final 5 mL concentrate obtained by carrying 250 mL aqueous standard containing 1 ppb of both 2,6- and 2,4-TDA through methylene chloride concentration procedure. LC conditions described in text; B, 5 µL GLC injection of TFAA derivative from 6.2-7.2 mL LC fraction collected in A; C, 5 µL GLC injection of TFAA derivative from 9.4-10.4 mL LC fraction collected in A. GLC conditions described in text.

Derivatization and GLC.—Three drops of a solution of 0.04M NaOH, 0.005M NaH_2PO_4, and 0.015M Na_2HPO_4 was added to each vial containing an LC fraction. Each fraction was extracted with 500 µL ethyl acetate in the capped vial. After the 2 layers were allowed to separate, a 1 mL syringe (blunt end No. 22 gauge needle) was inserted to the bottom of the tapered 3 mL mini-vial and the aqueous layer was withdrawn and discarded. TFAA (100 µL) was added to the ethyl acetate layer remaining in the vial. The vial was capped, shaken, and allowed to stand 10 min. Nitrogen was used to evaporate the sample to dryness. Any TDA-TFAA derivative present was then redissolved in ethyl acetate: retorted pouch samples, 100 µL; boil-in-bag samples, 50 µL.

Aliquots (5 µL) of these final ethyl acetate solutions were injected into the gas chromatograph and 2,4- and/or 2,6-TDA (derivatives) were quantitated by the external standard technique. Standards used to calibrate the gas chromatograph were prepared as follows: A stock solution was made by adding ca 10 mg (weighed to 0.01 mg) of both 2,4- and 2,6-TDA to a 100 mL volumetric flask. TFAA (1.0 mL) was added and mixed, and the solution was allowed to stand 30 min. The resulting TDA-TFAA derivatives were dissolved in and diluted to volume with ethyl acetate. This stock solution was diluted, using ethyl acetate, to obtain the appropriate ppb (µg/L) standard solutions for injection into the gas chromatograph. The measured TDA-TFAA GLC responses were expressed as the equivalent TDA concentration.

GLC/MS.—Aliquots (10 µL) of derivatized ethyl acetate isolates were injected into the GLC/MS instrument and the data were acquired as outlined under *Apparatus* (**h**). The MID chromatograms and MID background-subtracted spectra obtained were compared with standards analyzed on the same day.

Results and Discussion

Aromatic amines such as TDA typically exhibit strong adsorptive characteristics when analyzed by GLC. This results in poor or even unacceptable chromatography. Treatment of the GLC

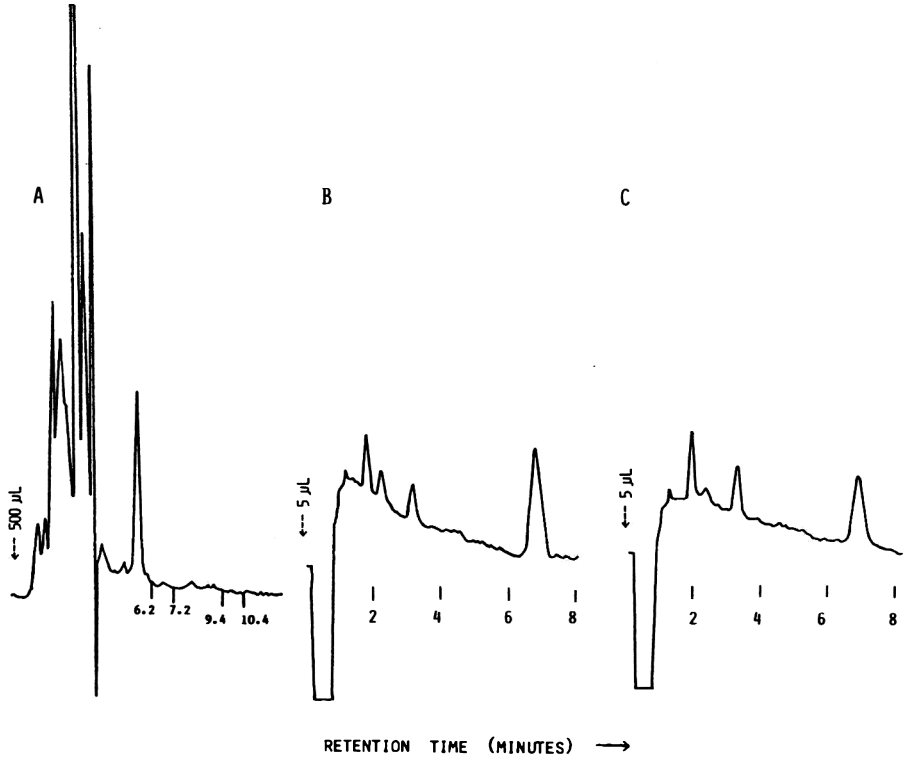


Figure 2. Reagent blanks: A, 500 µL LC injection of final 5 mL concentrate obtained by carrying 250 mL distilled water through methylene chloride concentration procedure; B, 5 µL injection of TFAA derivative from 6.2-7.2 mL LC fraction collected from A; C, 5 µL GLC injection of TFAA derivative from 9.4-10.4 mL LC fraction collected in A. LC and GLC conditions as described in Figure 1.

column packing material, or the column wall in the case of capillary GC, with base has been shown to effectively minimize these adsorptive interactions (5-7). Derivatization of amines prior to GLC has also been shown to be an effective means of minimizing these adsorptive interactions while often improving the stability and volatility of the amine (8-10). The most commonly prepared derivatives of primary amines are generally formed by acylation with either acid anhydrides or acid halides (11-13). Other derivatives of amines have also been reported involving silylation (14), Schiff's base formation (15), 2,4-dinitrophenylation (16), and alkylation (17).

In this study, derivatization was the preferred choice because TDA was adsorbed by both the GLC column packing material and the GLC/MS interface. Quantitative derivatization of TDA was rapidly achieved by reacting with TFAA. The TDA-TFAA derivative minimized adsorption problems and was much more stable than the free amine. The thermionic N/P detector was very responsive to the TDA-TFAA derivatives, thereby maximizing both sensitivity and specificity.

Operating the gas chromatograph isothermally at 210°C with a carrier gas (nitrogen) flow rate of 20 mL/min resulted in retention times for 2,6- and 2,4-TDA-TFAA derivatives of 3.8 and 4.2 min, respectively. Although baseline resolution between 2,6- and 2,4-TDA-TFAA was not achieved, this posed no problem because the compounds were completely separated by LC and therefore were never both present in the same GLC injection.

LC and GLC chromatograms depicting the LC fractionation and subsequent GLC confirmation of 2,4- and 2,6-TDA are shown in Figures 1A, B, and C. Figure 1A shows the LC chromatogram obtained for a 500 µL injection of a standard initially containing 1 ppb of both 2,6- and 2,4-TDA that was concentrated 50-fold. The two 1 mL fractions collected that correspond to the 2,6- (6.2-7.2 mL) and 2,4-TDA (9.4-10.4 mL) peaks are indicated. Both these fractions were partitioned into ethyl acetate and then reacted with TFAA. Figures 1B and C are the GLC chromatograms

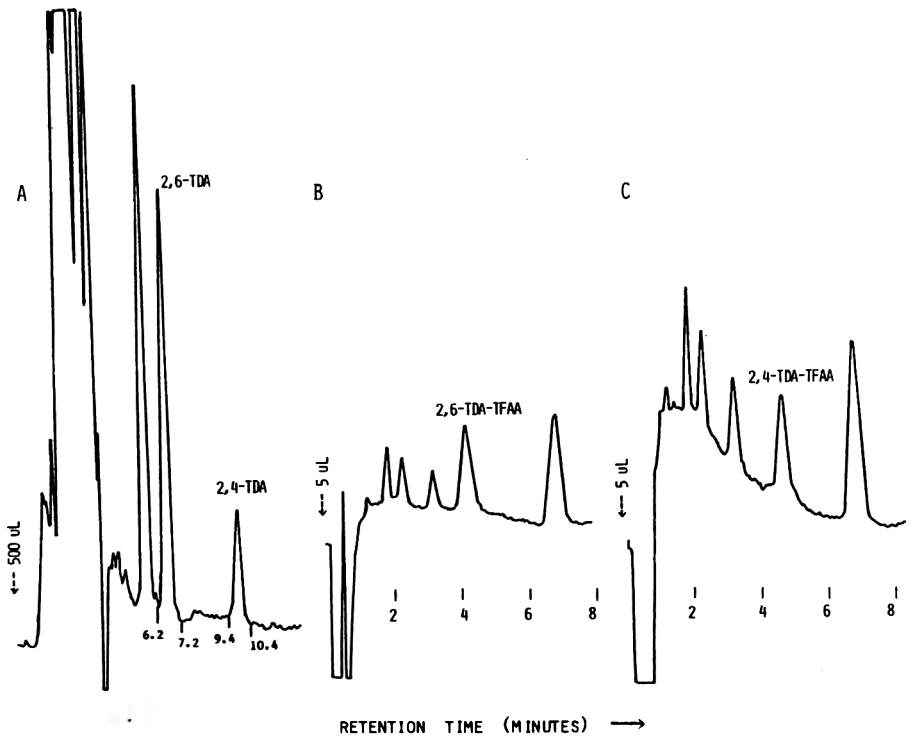


Figure 3. A, 500 μL LC injection of final 5 mL concentrate obtained by carrying 250 mL retortable pouch sample extract B through methylene chloride concentration procedure; 5 μL GLC injection of TFAA derivatives formed from (B) 6.2-7.2 mL and (C) 9.4-10.4 mL LC fractions collected from A. LC and GLC conditions as described in Figure 1.

corresponding to the 2,6- and 2,4-TDA-TFAA derivatives, respectively, obtained by injecting 5 μL of these derivatized LC fractions into the gas chromatograph. Figures 2A, B, and C are LC and GLC chromatograms for the reagent blanks. These were obtained in the same manner described for Figures 1A, B, and C, but using a 50-fold distilled water concentrate.

A series of 2,6- and 2,4-TDA standards ranging in concentration from 5 to 80 ppb were injected into the liquid chromatograph; the appropriate fractions were collected, partitioned into ethyl acetate, derivatized, and injected into the gas chromatograph. The concentration of TDA observed was quantitated by the external standard technique. A linear least squares fit of LC vs GLC concentrations forced through the origin was calculated. The concentrations of any 2,4- and 2,6-TDA measured in the retortable pouch and boil-in-bag extracts by GLC were related to the concentrations measured by LC through the use of these calibration curves.

The LC vs GLC concentration calibration used for the boil-in-bag extracts, based on duplicate 200 μL LC injections of TDA standards ranging from 5 to 80 ppb, was plotted. The slope of this calibration for 2,4-TDA was 2.8 ($y = mx$) with a linear correlation coefficient of 0.985 and relative standard error of ±8.3% ($n = 10$). Similarly, the slope for 2,6-TDA was 4.0 with a linear correlation coefficient of 0.998 and relative standard error of ±2.3% ($n = 10$). For the retortable pouch extracts a similar LC vs GLC concentration calibration was prepared based on single 500 μL LC injections.

The sample extracts were all concentrated from 250 to 5 mL before injection into the liquid chromatograph. This 50-fold concentration, considered along with the LC to GLC calibration, resulted in establishing a lower TDA detection limit for GLC (10 mm peak) at 0.2 ppb in the original sample extracts. For LC the lower detection limit was 0.1 ppb.

Four different retortable pouches and 5 varieties of boil-in-bags were each extracted and analyzed for any migration of TDA. The concentrations of 2,4- and 2,6-TDA measured in these extracts by both the LC and GLC procedures (single determinations) are shown in Table 1. All 4 of the retortable pouch extracts contained

Table 1. Concentration (ng/L) of 2,4- and 2,6-TDA in aqueous extracts of retortable pouches and boil-in-bags, determined by LC and GLC

	2,4-TDA		2,6-TDA	
Sample	LC	GC	LC	GC
	Retortable Pouches[a]			
A	2.0	1.9[b]	1.4	1.7[b]
B	0.6	0.5[b]	1.4	0.9[b]
C	1.6	2.0[b]	2.2	1.2
D	0.7	0.9[b]	0.5	0.7[b]
	Boil-in-Bags[c]			
E	<0.1	<0.2	<0.1	<0.2
F	<0.1	<0.2	0.1	<0.2
G	0.2	<0.2	0.1	<0.2
H	0.9	0.4	0.7	0.7
I	0.3	0.3	0.4	0.4

[a] 4 mL/6.4 sq. cm, 121°C, 2 h.
[b] Confirmed by MID GLC/MS.
[c] 2.5 mL/6.4 sq. cm, 100°C, 2 h.

measurable levels of both 2,4- and 2,6-TDA, ranging from 0.5 to 2.2 ppb. The boil-in-bag extracts, on the other hand, exhibited much lower levels of TDA migration ranging from none detected (<0.1) to 0.9 ppb. Overall, the agreement between the LC and GLC measured concentration is good, with an average difference of ±0.3 ppb.

Figures 3A, B, and C are representative LC and GLC chromatograms, similar to those described in Figure 1, obtained from one of the retortable pouch extracts (Sample B).

As a means of substantiating the GLC confirmatory technique, the 4 retortable pouch extracts were also analyzed by GLC/MS. Sufficient sensitivity to confirm the presence of TDA at concentrations as low as 0.5 ppb in the unconcentrated aqueous extracts was achieved using MID GLC/MS.

Full mass scan spectra of the TDA-TFAA derivatives revealed a number of ions potentially useful for MID GLC/MS, while retaining a high level of specificity for the particular compound. The ions chosen correlated with the following presumed fragmentation: m/z 314 $(M)^{+\cdot}$, 245 $(M - CF_3)^+$, 243 $(M - CF_3 - H_2)^+$, 148 $(M - CH_3 - CF_3CO\cdot)^{+\cdot}$, and 104 $(M - 210)^{+\cdot}$. The ion at m/z 104 occurred in the spectra from both the derivatized and underivatized TDA. It presumably involved the loss of both trifluoroacetyl groups plus the loss of one amino group. A sixth ion at m/z 69, although useful in preliminary studies involving standards, was subject to relatively high background levels in the samples and was not used in the confirmation.

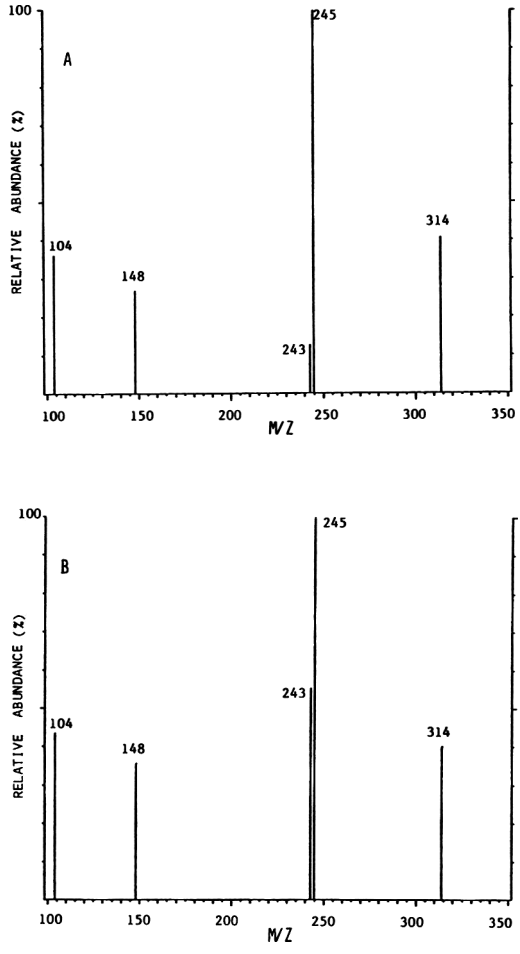


Figure 4. A, MID mass spectra (10 μL injection) of TFAA derivative obtained by collecting 2,4-TDA LC fraction (9.4-10.4 mL) from retortable pouch extract sample C; B, same as A but for 2,6-TDA LC fraction (6.2-7.2 mL) from retortable pouch extract Sample D. Conditions and procedures described in text.

The MID mass spectra of the 2,4- and 2,6-TDA-TFAA derivatives differed primarily in the relative abundance of m/z 243 (about 10 and 50%, respectively, with m/z 245 at 100%). Representative MID spectra for 2,4- and 2,6-TDA-TFAA derivatives are shown in Figures 4A and B, respectively. Generally, 4 spectra were summed for the MID spectra with 2 spectra from each side of the GLC peak chosen as background in a manner consistent for samples and standards. The relative abundances of ions at m/z 104, 148, 243, 245, and 314 between spectra of samples and standards generally agreed within ±10%.

Conclusion

An LC/GLC technique for confirming the presence of 2,4- and/or 2,6- TDA in aqueous extracts is described. Good precision was obtained as exhibited in the LC vs GLC calibrations. The GLC/MS findings substantiated the validity of this technique, the utility of which was demonstrated in its application to aqueous extracts of several retortable pouches and boil-in-bags, shown to have concentrations of 2,4- and 2,6-TDA ranging from <0.1 to 2.2 ppb.

References

(1) Hawley, G. G. (1971) *The Condensed Chemical Dictionary*, 8th Ed., Van Nostrand Reinhold, New York, NY, p. 989
(2) Ito, N., Hiasa, Y., Konishi, Y., & Marugami, M. (1969) *Cancer Res.* **29,** 1137–1145
(3) Weisberger, E. K., *et al.* (1978) *J. Environ. Pathol. Toxicol.* **2,** 325–356
(4) Snyder, R. C., & Breder, C. V. (1982) *J. Chromatogr.* **236,** 429–440
(5) Choudhary, G. (1980) *J. Chromatogr.* **193,** 277–284
(6) Olufsen, B. (1979) *J. Chromatogr.* **179,** 97–103
(7) Becher, G. (1981) *J. Chromatogr.* **211,** 103–110
(8) Blau, K., & King, G. (Eds) (1978) *Handbook of Derivatives for Chromatography*, Heyden, London, Chapter 3
(9) Metcalfe, L. D. (1975) *J. Chromatogr. Sci.* **13,** 516–519
(10) Vanden Heuvel, W. J. A., Gardiner, W. L., & Horning, E. D. (1964) *Anal. Chem.* **36,** 1550–1560
(11) Martin, I. L., & Ansel, G. B. (1973) *Biochem. Pharmacol.* **22,** 521–533
(12) Irvine, W. J., & Saxby, M. J. (1969) *J. Chromatogr.* **43,** 129–132
(13) Wilkinson, G. R. (1970) *Anal. Lett.* **3,** 289–298
(14) Piekos, R., Teodorczyk, J., Grzybowski, J., Kobylczyk, K., & Osmialowski, K. (1976) *J. Chromatogr.* **117,** 431—435
(15) Lhuguenot, J. C., & Maume, B. F. (1974) *J. Chromatogr. Sci.* **12,** 411–418
(16) Cohen, I. C., & Wheals, B. B. (1969) *J. Chromatogr.* **43,** 233–240
(17) Thenot, J. P., & Horning, E. C. (1972) *Anal. Lett.* **5,** 519–529

ALCOHOLIC BEVERAGES

High Performance Liquid Chromatography with Fluorescence and Ultraviolet Detection of Polynuclear Aromatic Hydrocarbons in Barley Malt

FRANK L. JOE, JR, JEAN SALEMME, and THOMAS FAZIO
Food and Drug Administration, Division of Chemistry and Physics, Washington, DC 20204

A simple, rapid method has been developed for the separation and determination of polynuclear aromatic hydrocarbons (PAHs) in barley malt. An ultrasonic-cyclohexane extraction method was used to separate the PAHs from ground barley malt. The cyclohexane extracts were purified by chromatography through a water-deactivated silica gel-alumina column. The eluate from the column was concentrated and purified further by partitioning between dimethyl sulfoxide (DMSO) and cyclohexane. The DMSO extract was diluted with water and the PAHs were extracted back into cyclohexane. The cyclohexane extract was washed with water, dried through sodium sulfate, and evaporated, and the resulting residue was dissolved in 80% aqueous acetonitrile-methanol (1 + 1) and subjected to reverse phase high performance liquid chromatography. Thirty barley malt samples were analyzed using this procedure. Peaks having the same retention time as the carcinogen benzo(a)pyrene were isolated from 18 of the samples, and were equivalent to trace levels ranging from <0.1 to 0.2 ppb. Average recoveries of 11 PAHs, including benzo(a)pyrene, benzo(b)fluoranthene, indeno(1,2,3-cd)pyrene, and benz(a)anthracene, added to 25 g samples at 2.5 and 5 ppb, ranged from 78 to 97%, with a mean relative standard deviation of 6.6%.

Industrial development has unfavorably altered the natural balance between production and degradation of polynuclear aromatic hydrocarbons (PAHs); consequently, the accumulation of PAHs is increasing (1). Although PAHs are formed from both natural and anthropogenic sources, the latter are by far the major contributors (2). PAHs enter into the environment through many routes, including contamination from combustion products of fossil fuels, oil spills, effluents from industrial processes, and fallout from the atmosphere (3). The carcinogenic and mutagenic properties of many PAHS are well established (4–8); thus there is continuing concern about the contamination of many substances with PAHs, especially air, water, and foods (9). This investigation focuses on the latter.

Foods such as barley, which have been processed using fossil fuels, e.g., fuel oils, are of special concern because these fuels contain many hydrocarbons (10). Although formerly used extensively as a cereal food, barley is now used primarily for brewing beer. Two procedures, direct and indirect firing, are used for kiln-drying barley before it is used in the malting process; the former is used primarily in North America (11). Direct heating of grains with fossil fuels leads to higher levels of PAH contamination (11,12). This seems probable since Hutt et al. (11) have shown that, during the process of direct kiln-drying of grains such as barley, all combustion gases produced pass through the grain column.

Methods for isolating and measuring various PAHs in different matrices cover a broad area and involve a variety of techniques. In the majority of samples of interest, a complex array of other organic compounds accompany the PAHs; consequently, without extensive cleanup, successful determination is almost impossible. During the initial isolation steps, several techniques have been employed involving Soxhlet extraction, saponification, liquid-liquid extraction, ultrasonic vibration (sonication), and shredding (13–21). Each of these techniques has its advantages; however, sonication has proved useful in rapidly separating PAHs from automobile exhaust (15), atmospheric particles (16, 17), and sediments (18). Lankmayr et al. (17) and Tan (18) compared sonication to Soxhlet extraction and concluded that the former was as efficient in isolating PAHs from the sample matrix as the latter and superior in regard to the separation time. Although these investigators isolated PAHs from matrices which are very different from the complex composition of foodstuffs, we felt that sonication might be a rapid, efficient, and reliable method for the quantitative

Received February 17, 1982. Accepted March 30, 1982.
This paper was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

0004-5756/82/6506-1395–08$01.00

determination of PAHs in certain foods which do not require saponification or Soxhlet extraction.

The feasibility of utilizing ultrasonics to rapidly separate PAHs from barley malt matrices was investigated along with the use of smaller sample portions for conducting analyses. In the suggested method, the nonvolatile hydrocarbon components, e.g., PAHs, are extracted from the ground barley malt by sonication in the presence of cyclohexane, cleaned up by column chromatography and liquid-liquid partitioning, and finally analyzed by reverse phase high performance liquid chromatography (HPLC) with ultraviolet (UV) and fluorescence detection. This procedure provides the sensitivity necessary to determine low levels of PAHs in barley malt that has been subjected to direct kiln-drying using fossil fuels.

METHOD

Apparatus

Clean all glassware with chromic acid cleaning solution, rinse with distilled water, oven-dry at 180°C, and solvent-rinse before use.

(a) *Centrifuge bottle.*—500 mL with ⅋ 24/40 joint (K-294260, Kontes Glass Co., Vineland, NJ 08360).

(b) *Ultrasonic vibrator.*—37.8 L capacity, Branson DHA-1000 (American Scientific Products, 8855 McGaw Rd, Columbia, MD 21045).

(c) *Aspirator assembly.*—Parts include 500 mL Erlenmeyer flask, with ⅋ 24/40 outer joint (Kontes K-617000), and ⅋ 24/40 stopper (see Figure 1) having inlet and outlet tube fused into stopper (Kontes K-331751). Stopper is similar to that used in wash bottle assembly except outlet tube, which connects to vacuum line, is bent downward at 45° angle to prevent flowback of condensate into flask; inner part of inlet tube, which allows for suction of solution of interest, is cut off ca 2 cm below 24/40 joint; and outer part is cut off 4 cm from its constricted end, sealed to 30 cm tube of similar diameter (30 cm tube should be constricted at end opposite seal), and bent downward at ca 130° angle ca 2 cm below seal. Aspirator assembly is used by attaching outlet tube to vacuum line and immersing constricted end of inlet tube in solution to be aspirated. After solution transfer is completed, inlet tube is rinsed as described under *Extraction*.

(d) *Chromatographic column.*—14.5 × 250 mm with ⅋ 24/40 joint.

(e) *Reservoir.*—250 mL, equipped with ⅋ 24/40 upper and lower joints.

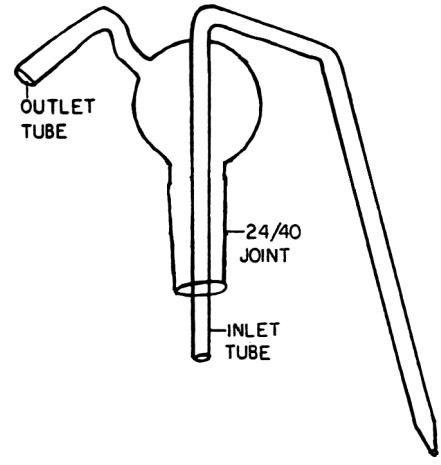


Figure 1. Aspirator assembly stopper.

(f) *Flasks.*—Pear-shaped, 300 and 500 mL with ⅋ 24/40 joint (Kontes, K-294260).

(g) *Flash evaporator.*—Buchler PF-9GN, equipped with temperature-controlled water bath (Buchler Instruments, Fort Lee, NJ 07024).

(h) *Separatory funnels.*—125 and 500 mL, equipped with Teflon stopcocks.

(i) *Filter funnel.*—Buchner, 60 mL, with coarse porosity fritted disk (Kontes K-955000).

(j) *Concentrator tubes.*—Kimax, 15 mL conical, graduated 0.1 mL subdivisions (American Scientific Products).

(k) *Sample clarification kit.*—Parts include 3 mL Luer-Lok syringe and 13 mm Swinny stainless steel disk filter holder (XX30-012-00, Millipore Corp., Bedford, MA 01730), with 0.2 μm filter (FGLP 01300, Millipore Corp.).

(l) *High performance liquid chromatograph.*—Model 332 programmable gradient system (Altex Scientific Co., Inc., Berkeley, CA 94710). *Column.*—4.6 mm × 25 cm Zorbax ODS (DuPont Co., Analytical Instruments Div., Wilmington, DE 19898). *Detectors.*—Model SF-770 UV-Vis detector (Schoeffel Instruments Div., Westwood, NJ 07675) set at 289 nm; and Model 970 fluorometer (Schoeffel) set at 333 nm (excitation filter 7-54; emission filter KV-370) in series with UV detector. (Note: 7-54 excitation filter has broad transmission range with maximum at 310 nm; emission filter (KV-370) has 10^{-3}% transmission at 340 nm and 99% transmission at 390 nm.) Operating parameters.—Mobile phase: reservoir A contains water and reservoir B contains methanol–acetonitrile (1 + 1); flow rate 1 mL/min; injection volume 20 μL; gradient program—Segment 1: 80% B-100% B in 20 min; Segment 2: hold 100% B for 20 min; Segment 3

(equilibration of column): 100% B-80% B in 5 min, hold at 80% B for 20 min before next injection.

Reagents

(a) *Solvents.*—Cyclohexane, acetone, acetonitrile, and methanol. Distilled-in-glass grade-spectrograde (Burdick and Jackson Laboratories, Inc., Musgekon, MI 49442).

(b) *Dimethyl sulfoxide (DMSO).*—Spectrophotometric grade (J.T. Baker Co., Phillipsburg, NJ 08865). Equilibrate with cyclohexane.

(c) *Solvent B.*—Acetonitrile-methanol (1 + 1).

(d) *Solvent C.*—Solvent B-water (80 + 20).

(e) *Deionized water.*—Extract 2 L deionized water with two 100 mL portions of cyclohexane to remove impurities.

(f) *PAH standards.*—Phenanthrene (Phen), fluoranthene (F), pyrene (Py), benz(a)anthracene (BaA), benzo(b)fluoranthene (BbF), benzo(e)pyrene (BeP), benzo(a)pyrene (BaP), dibenz(a,h)anthracene (DBahA), benzo(b)chrysene (BbCh), indeno(1,2,3-cd)pyrene (IcdPy), benzo(g,h,i)perylene (BghiP), dibenzo(a,i)pyrene (DBaiP), and coronene (Cor), obtained from Division of Chemistry and Physics, Food and Drug Administration, Washington, DC.

(g) *Standard solutions.*—(*1*) *Stock solutions.*—0.1 mg/mL. Dissolve 1 mg each PAH standard in 10 mL benzene. (DBaiP may require heating to dissolve.) (*2*) *Working standards.*—0.25 μg/mL. Pipet 0.25 mL of each stock solution (except BbCh) into 100 mL volumetric flask and dilute to volume with solvent B. Use solvent C to prepare 0.25 μg/mL BbCh solution separately. (*3*) *Internal standard reference solution.*—Pipet 1 mL working standard PAH mixture into concentrator tube and concentrate just to dryness under gentle stream of nitrogen using 30°C water bath. Add 1 mL BbCh solution (0.25 μg/mL solvent C) and sonicate 1 min. Inject 20 μL for HPLC.

(h) *Alumina.*—Aluminum oxide 90. Active neutral (activity stage I); particle size 0.063–0.200 mm (70–230 mesh ASTM) (E. Merck, Darmstadt, Germany, obtained from Brinkmann Instruments, Inc., Westbury, NY 11590, No. 1077). Deactivate as described in ref. 10.

(i) *Silica gel 60.*—Particle size 0.063–0.200 mm (70–230 mesh ASTM) (Brinkmann Instruments, Inc., No. 7734). Deactivate as follows: Heat portion of silica gel in tared Erlenmeyer flask in 160°C oven for 16 h (overnight). Heat tared glass stopper in separate beaker along with flask containing silica gel. Remove flask from oven, stopper immediately, and cool in desiccator. Weigh flask to determine weight of dried silica gel. Add enough water to dried silica gel so that added water accounts for 15% by weight of deactivated silica gel. Immediately stopper flask and shake vigorously 15 min. Store ≥4 h before use. (Note: Test deactivated silica gel and alumina as indicated under *Column Chromatography.*)

Column Chromatography

Add 5 g silica gel (deactivated with 15% water), 5 g alumina (deactivated with 10% water), and 10 g Na_2SO_4, in that order, to 14.5 × 250 mm column containing glass wool plug by tapping gently during each addition. Fit column with 250 mL reservoir. Wash column with 50 mL cyclohexane. Stop flow when liquid level just reaches top of Na_2SO_4 bed. Test before use as follows. Prepare 2 columns as described above (1 column for blank and 1 for recovery). Add 75 mL cyclohexane to 1 column and 75 mL cyclohexane spiked with 0.25 mL of a PAH mixture containing 0.25 μg/mL each of Phen, F, Py, BaA, BbF, BeP, BaP, DBahA, IcdPy, BghiP, DBaiP, and Cor (equivalent to 2.5 ppb spike for 25 g sample) to other column. Let solvent percolate through blank and spike columns. Collect eluates in separate 500 mL pear-shaped flasks. Repeat column elution with three 75 mL portions of cyclohexane. Let each eluate drain just to top of Na_2SO_4 bed before adding succeeding eluates. Evaporate combined cyclohexane solutions to ca 5 mL, using flash evaporator with 40°C water bath. Transfer concentrates to 15 mL concentrator tubes with disposable Pasteur type pipets. Rinse each flask with three 1 mL portions of cyclohexane and transfer to proper concentrator tube. Place tubes in 30°C water bath and concentrate solutions to dryness under gentle stream of nitrogen. Add 0.25 mL BbCh solution (0.25 μg/mL) in solvent C and subject to ultrasonic vibration for ca 3 min. (If necessary, filter solutions with sample clarification kit into sample vial.) Subject to HPLC. Solvents must be free of interfering peaks, and recovery for each PAH should be ≥90%.

Extraction

Place ca 100 g barley malt in blender jar and homogenize 3 min. Weigh 25 g homogenized sample into 500 mL centrifuge bottle. Add 75 mL cyclohexane, stopper, shake vigorously 15 s, and place in ultrasonic bath 15 min. Shake barley malt sample vigorously every 4 min since barley malt tends to settle while in ultrasonic bath. Centrifuge at 2000 rpm 15 min. Carefully

aspirate off top layer, using aspirator assembly, into 500 mL Erlenmeyer flask. Rinse inlet tube with 10 mL cyclohexane and also collect in flask. Transfer extract to silica gel–alumina column prepared as indicated under *Column Chromatography*. Rinse Erlenmeyer flask with two 5 mL portions of cyclohexane and combine with previous extract in column. Collect eluate in 500 mL pear-shaped flask. Repeat extraction of sample, centrifugation, aspiration of top layer, rinsing operations, and addition of extract and rinses to column 2 more times.

After third extract and rinses drain just to top of Na_2SO_4, add 75 mL cyclohexane and also collect this eluate in flask. Evaporate combined eluates (ca 355 mL) to ca 25 mL, using flash evaporator with 40°C water bath. Transfer eluate to 125 mL separatory funnel containing 15 mL DMSO pre-equilibrated with cyclohexane. Quantitatively rinse flask with 25 mL cyclohexane and combine with concentrate in 125 mL separatory funnel. Shake funnel vigorously 2 min. Let layers separate. Drain bottom DMSO layer into second 125 mL separatory funnel containing 15 mL cyclohexane pre-equilibrated with DMSO. Shake second funnel 30 s. After layers separate, drain DMSO layer into 500 mL separatory funnel containing 90 mL deionized water (see *Reagents* (e)) and 15 mL cyclohexane. Repeat extraction with 2 additional 15 mL portions of DMSO, and combine DMSO extracts in 500 mL separatory funnel. Shake 500 mL separatory funnel vigorously 2 min and let layers separate. Draw off lower aqueous phase into second 500 mL separatory funnel containing 15 mL cyclohexane. Repeat extraction. After layers separate, draw off bottom aqueous phase and discard. Combine 2 extracts in first 500 mL funnel. Rinse second funnel with two 10 mL portions of cyclohexane and transfer each rinse to first funnel. Wash combined extracts with 100 mL deionized water (see *Reagents* (e)), shaking wash 1 min. Let phases separate and discard lower aqueous phase.

Add 50 g Na_2SO_4 to 60 mL Buchner funnel with coarse fritted disk. Rinse Na_2SO_4 with 50 mL cyclohexane, letting liquid drain by gravity. Discard wash. Filter combined cyclohexane extracts through Na_2SO_4 bed into 300 mL pear-shaped flask. Rinse separatory funnel with two 10 mL portions of cyclohexane and pass each through Na_2SO_4 bed into flask. Evaporate combined extracts and washes to 3–5 mL, using flash evaporator with 40°C water bath. Using disposable Pasteur pipet, transfer concentrate to 15 mL concentrator tube. Rinse flask with three 1 mL portions of cyclohexane and transfer each to concentrator tube. Place tube in 30°C water bath and concentrate contents to dryness under gentle stream of nitrogen. Add 0.25 mL BbCh (0.25 μg/mL) in solvent C and subject to ultrasonic vibration ca 3 min. Filter if necessary and save for HPLC.

High Performance Liquid Chromatography

Inject 20 μL sample extract or standard solutions onto Zorbax ODS column and follow solvent program as described under *Apparatus* (1). Between injections, flush sample loop and port passages with ca 3 mL solvent B to prevent cross-contamination. Compare retention times of any peaks observed with those obtained for known PAH standards chromatographed under same conditions. Inject standards after every third sample. Calculate quantity of PAH in sample extracts by internal standard procedure.

Calculation

Calculate levels of PAHs found in sample extracts as follows:

$$\text{PAH, ppb} = (F \times (Ru/Ris) \times IS \times (Vd/Vi))/W$$

where F = response of internal standard divided by response of PAH as obtained from analysis of standard mixture; Ru = response of given PAH in sample extract; Ris = response of internal standard added to same sample extract; IS = internal standard injected, ng; Vd = dilution volume, μL, sample extract; Vi = volume, μL, sample extract injected; and W = weight, g, of sample taken for analysis.

Results and Discussion

The barley malt samples used in this investigation were part of a group collected during the Food and Drug Administration's nationwide survey of domestic and imported malts for nitrosamine contamination (22). Of 196 samples collected, 30 were selected randomly for PAH analysis. The PAHs were measured with UV and fluorescence detectors connected in series, with the former set at 289 nm and the latter set at an excitation wavelength of 333 nm. The use of these wavelengths provided both sensitivity and selectivity for measurement of PAHs.

The objectives of this study were to develop a rapid procedure for the initial separation of PAHs from the sample matrix and to develop a method which was applicable to relatively small sample portions. To accomplish these objectives, 3 extraction procedures were investigated: (*1*)

Table 1. Determination of PAHs (ppb) in barley malt by different methods of analysis

Method	F	BbF	BeP	BaP	BghiP
FDA direct digestion	0.2	0.1	1.2	0.2	0.1
Soxhlet extraction	0.2	0.1	1.1	0.2	0.1
Ultrasonic technique	0.2	0.1	1.0	0.2	0.1

Soxhlet extraction with alcoholic KOH: 25 g barley malt was thoroughly mixed with 100 g Na_2SO_4 and Soxhlet-extracted for 4 h with ethanol. KOH was added to the boiling flask to simultaneously saponify the extract. This procedure was a modification of that of Howard et al. (23) for determining PAHs in smoked foods. (2) Direct saponification of the sample: 25 g sample was added directly to the boiling flask along with 6 g KOH and 100 mL ethanol and digested for 2.5 h. This procedure was similar to that employed by Joe et al. (24) for the analysis of root vegetables. (3) Sonication as described here. To obtain a direct correlation between the data obtained from each procedure, the same unfortified barley malt sample was analyzed by the 3 procedures. The results are summarized in Table 1, where the levels found were approximately the same for each procedure. Considering the various aspects associated with carrying out each procedure, it was concluded that the sonication technique was as reliable as the other 2 procedures and less time-consuming than either. For example, with the sonication procedure, 4 samples could be prepared for HPLC in approximately 8 h, whereas with the other 2 procedures, from 1.5 to 3 days were required to reach a similar point.

Table 2. Fluorescence recovery (%) of PAHs from barley malt samples[a]

	2.5 ppb level		5 ppb level	
PAH	Av.	CV[b]	Av.	CV
Phen[c]	—		—	
F	86	2.3	79	7.7
Py	81	1.4	82	6.5
BaA	78	4.4	82	6.0
BbF	85	7.5	88	6.0
BeP	86	8.4	97	12.4
BaP	83	6.0	85	7.2
DBahA	86	6.7	87	7.0
BbCh	—	—	—	—
IcdPy	85	10.6	87	8.1
BghiP	81	8.0	85	5.9
DBaiP	79	7.8	89	2.6
Cor	83	9.7	87	3.5
Mean		6.6		6.6

[a] Triplicate determinations; excitation at 333 nm.
[b] CV = coefficient of variation or relative standard deviation.
[c] Interference peak from samples prevented reliable determination of Phen.

Table 3. Relative fluorescence and UV response factors of PAHs vs BbCh[a]

Compound[b]	Fluorescence (ex: 333 nm)	UV (289 nm)
Phen	—	3.18
F	1.08	6.20
Py	1.31	6.89
BaA	2.58	2.38
BbF	0.70	1.88
BeP	6.25[c]	2.43
BaP	1.17	1.11
DBahA	1.27	0.34
BbCh	1.00	1.00
BghiP	2.99	1.02
DBaiP	4.63	1.63
Cor	6.95	1.35

[a] $F = (R_{BbCh} / R_{PAH})$.
[b] 5 ng of each PAH was injected.
[c] As noted in text, fluorescence factor for BeP varied somewhat from run to run.

To further establish the reliability of the sonication extraction technique, triplicate recoveries from the same barley malt sample at fortification levels of 2.5 and 5 ppb were conducted for 12 PAHs. The barley malt sample used for the recovery studies was found to contain less than 0.2 ppb of the PAHs investigated. The fluorescence data at the 333 nm excitation wavelength are shown in Table 2. These data show average recovery values ranging from 78 to 86% at the 2.5 ppb level, and from 79 to 97% at the 5 ppb level. Data for Phen are not included in Table 2 because Phen does not fluoresce at an excitation wavelength of 333 nm. The UV recovery data (not shown) were obtained at 289 nm and were in general greater than those obtained by fluorescence. This occurrence is probably attributable to 2 factors: (1) a shorter wavelength—289 nm for UV vs 333 nm excitation for fluorescence—is used in the UV, where more interfering compounds may absorb energy, and (2) many compounds which absorb in the UV region and act as interferences may not fluoresce and therefore are not measured by the fluorescence detector. In addition to the above recovery study, periodic recoveries of the aforementioned PAHs were conducted at the 5 ppb level with different barley malt samples to ensure that the sonication technique for isolation of PAHs from ground barley malt matrices was reliable. Furthermore, these results established that reliable analyses could be conducted on sample portions one-fourth the size used in previous studies (10, 24).

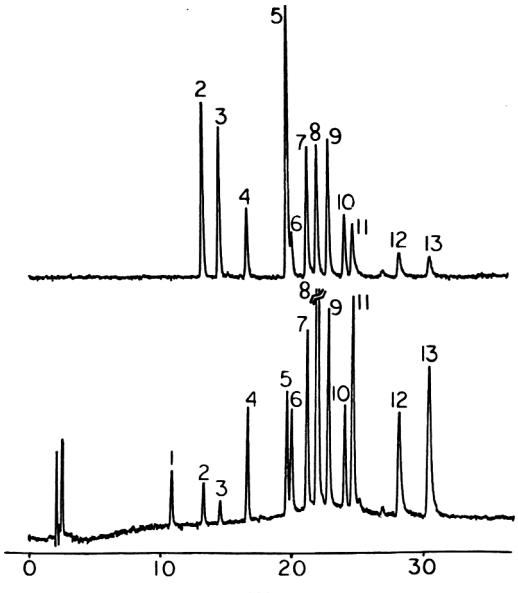


Figure 2. HPLC chromatograms for 5 ng of each PAH injected on column.

Top curve: fluorescence 333 nm, range 0.1 μA; bottom curve: UV 289 nm, attenuation 0.02 absorbance unit full scale (AUFS). 1, Phen; 2, F; 3, Py; 4, BaA; 5, BbF; 6, BeP; 7, BaP; 8, DBahA; 9, BbCh (internal standard); 10, IcdPy; 11, BghiP; 12, DBaiP; 13, Cor.

The flash evaporation apparatus used in this investigation did not cause any significant losses of any of the PAHs determined, including Phen. However, if more volatile PAHs, e.g., naphthalene, biphenyl, etc., are to be determined, another concentration procedure may be required. The Kuderna-Danish apparatus appears to be suitable. This apparatus was used to concentrate about 500 mL cyclohexane solution containing 1 μg each of several PAHs, including biphenyl and anthracene. An average of 95% of each PAH was recovered. The disadvantage of this technique is the time required to complete the concentration step, especially when a solvent such as cyclohexane is being evaporated. Usually, more than 1 h is required to evaporate about 500 mL of this solvent.

Several investigations were undertaken to reduce the time required to carry out an analysis. These studies included attempts to omit either the chromatographic column or the DMSO-cyclohexane liquid-liquid partitioning step. We found that if the silica gel-alumina column was omitted, severe emulsion problems resulted during the liquid-liquid partitioning steps. If the liquid-liquid partitioning steps were omitted, large background interferences were noted in the HPLC chromatograms. Thus, it was concluded that both cleanup steps were essential for efficient removal of extraneous materials.

Linearity studies using the Schoeffel fluorescence and/or UV detector have been reported for several PAHs by this author and others (10, 25). These data showed a linear response for the PAHs investigated over a wide concentration range. In this study, the stock solution internal standard procedure as described by Yost et al. (26) was employed to ascertain apparent levels of 12 PAHs. Typical response factors vs the internal standard (BbCh) are shown in Table 3. The data shown are for 5 ng of each PAH injected on the column, including the internal standard, BbCh. We found that the factor for BeP, as determined from fluorescence data, varied considerably from run to run. This variation appeared to be caused by 2 factors: (1) the lack of complete resolution (resolution = 0.8) between BeP and BbF; and (2) the wide difference between the magnitude of the fluorescence responses (about 7–1) of these 2 PAHs at 333 nm. In the UV chromatogram, however, the resolution is about 1 and the responses are approximately equal. As a result, data from the UV chromatograms were usually used to calculate the levels of BeP present in various sample extracts. Typical fluorescence and UV chromatograms of PAH standards are shown in Figure 2.

Fluorescence and UV chromatograms for a barley malt sample extract are shown in Figure 3. The PAHs indicated were measured by comparing the retention times of peaks in the sample chromatograms with those obtained in standard chromatograms. In these chromatograms, peaks are seen having retention times equivalent to those for F, Py, BaA, BbF, BeP, and BaP. A peak with the same retention time of Phen is seen in the lower UV chromatogram. The levels found in this particular sample ranged from 3.1 ppb for Phen to <0.1 ppb for BaP.

The data obtained for the 30 barley malt samples analyzed are shown in Table 4. The 5 PAHs most frequently found were F, Phen, BghiP, BeP, and BaP, in descending order. Of the 12 PAHs investigated, only Phen was found at levels greater than 5 ppb. Based on retention data, the PAHs BaA, BbF, BaP, DBahA, IcdPy, and DBaiP were all indicated at levels less than 0.2 ppb. Because of the low levels found, further confirmation of identity was not attempted. Generally, the levels of PAHs indicated in this study were within the ranges reported by previous investigators (11, 12). For example, the levels of

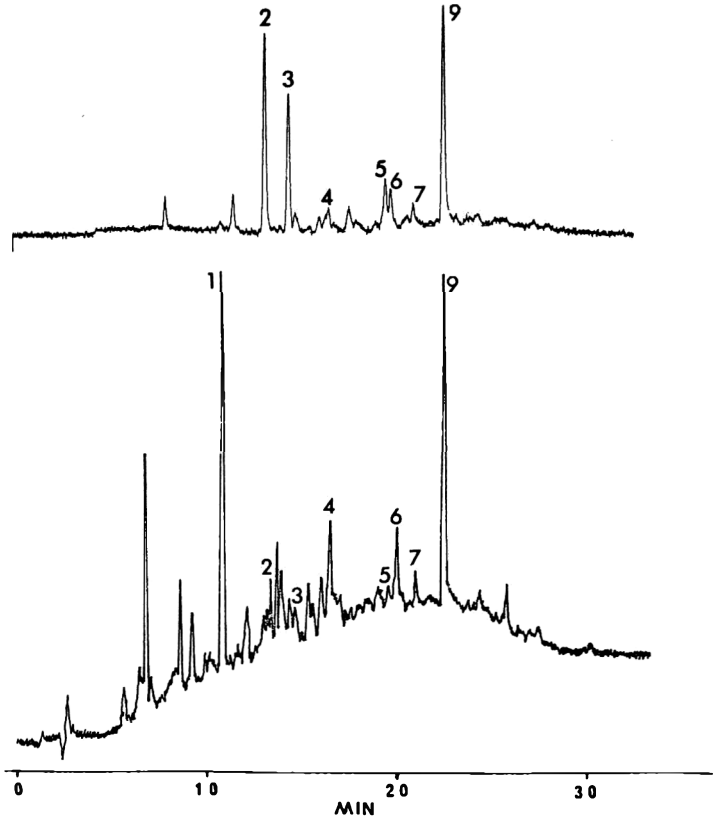


Figure 3. Representative HPLC chromatograms for unfortified barley malt sample.
Top curve: fluorescence 333 nm, range 0.1 μA; bottom curve: UV 289 nm, attenuation 0.02 AUFS. 1, Phen; 2, F; 3, Py; 4, BaA; 5, BbF; 6, BeP; 7, BaP; 8, DBahA; 9, BbCh (internal standard).

the 2 compounds, BaA and BaP, found in direct-heated corn by Hutt et al. (11), ranged from 0.06 to 0.47 ppb and 0.05 to 0.15 ppb, respectively, vs ≤0.1 to 0.3 ppb and <0.1 to 0.2 ppb for this study. These data seem to indicate that the kilning processes presently in use do not contribute significantly to the contamination of barley malt with PAHs during the drying procedures. Further indication of this can be noted from a previous FDA study (10) involving the analysis of 39 commercially available domestic and imported brands of beer. In that study, none of 4 PAHs (BaA, BaP, BghiP, and DBaiP) were apparent in any of the products down to a level of about 0.5 ppb. Nevertheless, further studies are required to more definitely assess the relationship of the fuels currently used in the direct kiln-drying process to the levels of PAHs found in the finished products.

Table 4. PAHs found in barley malt samples

Compound	No. of samples	Range, ppb	Av., ppb
Phen[a]	27	1.2–105	21
F	28	<0.1–2.7	0.4
Py	6	0.2–2.5	1.5
BaA	16	<0.1–0.3	0.2
BbF	14	<0.1–0.1	<0.1
BeP	21	0.2–1.7	0.9
BaP	18	<0.1–0.2	<0.1
DBahA	2	<0.1–0.1	0.1
IcdPy	3	<0.1–0.3	0.1
BghiP	24	<0.1–0.6	0.1
DBaiP	1	<0.1	<0.1
Cor	2	0.1–0.2	0.2

[a] Phen responses were usually incompletely resolved from interfering peaks.

Summary

A relatively rapid and reliable procedure has been developed for the analysis of barley malt for PAH contamination. Results obtained by the ultrasonic extraction procedure developed agreed favorably with results obtained by direct digestion and Soxhlet extraction. Low levels of PAH contamination were found in all the samples analyzed, with the most frequently found

PAHs being, in descending order, F, Phen, BghiP, BeP, and BaP. Although the carcinogen BaP was found at low levels, ≤0.2 ppb, it appeared to be present in over 50% of the samples analyzed.

REFERENCES

(1) Suess, M. J. (1976) *Sci. Total Environ.* **6,** 239–250
(2) Bartle, K. D., Lee, M. L., & Wise, S. A. (1981) *Chem. Soc. Rev.* **10,** 113–158
(3) Dunn, B. P. (1976) *Environ. Sci. Technol.* **10,** 1018–1021
(4) Shubik, P., & Hartwell, J. L. (1957) *Survey of Compounds Which Have Been Tested for Carcinogenic Activity,* Publ. No. 149, U.S.Public Health Service, Washington, DC
(5) Wynder, E. L., & Hoffmann, D. (1959) *Cancer* **12,** 1197–1199
(6) VanDuren, B. L., Sivak, A., Segal, A., & Orris, L. (1966) *J. Natl. Cancer Inst.* **37,** 519–526
(7) Hoffmann, D., Bomdimell, W. E., & Wynder, E. L. (1974) *Science* **183,** 215–216
(8) Mei-Teim, L., & Sandi, E. (1977) *Residue Rev.* **69,** 35–86
(9) Dunn, B. P., & Armour, R. J. (1980) *Anal. Chem.* **52,** 2027–2031
(10) Joe, F. L., Jr, Roseboro, E. L., & Fazio, T. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 641–646
(11) Hutt, W., Meiering, A., Oelschlager, W., & Winkler, E. (1978) *Can. Agric. Eng.* **20,** 103–107
(12) Winkler, E., Buchele, A., & Muller, O. (1977) *J. Chromatogr.* **138,** 151–164
(13) Lintas, C., & De Matthaels, M. C. (1979) *Food Cosmet. Toxicol.* **17,** 325–328
(14) Howard, J. W., & Fazio, T. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 1077–1103
(15) Nielsen, T. (1979) *J. Chromatogr.* **170,** 147–156
(16) Chatot, G., Castegnaro, M., Roche, J. L., & Fontanges, R. (1971) *Anal. Chim. Acta* **53,** 259–265
(17) Lankmayr, E. P., & Muller, K. (1979) *J. Chromatogr.* **170,** 139–146
(18) Tan, Y. L. (1979) *J. Chromatogr.* **176,** 319–327
(19) Steinheimer, T. R., Pereira, W. E., & Johnson, S. M. (1981) *Anal. Chim. Acta* **129,** 57–67
(20) Chesner, S. N., Gume, B. H., Hertz, H. S., May, W. E., & Wise, S. A. (1978) *Anal. Chem.* **50,** 805–810
(21) Golden, C., & Sawicki, E. (1976) *Anal. Lett.* **9,** 957–973
(22) Havery, D. C., Hotchkiss, J. H., & Fazio, T. (1981) *J. Food Sci.* **46,** 501–505
(23) Howard, J. W., Teague, R. T., White, R. H., & Fry, B. E., Jr (1966) *J. Assoc. Off. Anal. Chem.* **49,** 595–609
(24) Joe, F. L., Jr, Roseboro, E. L., & Fazio, T. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 615–620
(25) Das, B. S., & Thomas, G. H. (1978) *Anal. Chem.* **50,** 967–973
(26) Yost, R. W., Ettre, L. S., & Comlon, R. D. (1980) *Practical Liquid Chromatography—An Introduction,* Perkin-Elmer Corp., Norwalk, CT, pp. 230–234

ANTIBIOTICS

AC Polarographic Determination of Cloxacillin

LUIS J. NÚÑEZ-VERGARA, MARISOL M. SILVA, and JUAN A. SQUELLA
University of Chile, Department of Pharmacological Sciences and Formative Sciences, Faculty of Basic and Pharmaceutical Sciences, PO Box 233, Santiago-1, Chile

Hydrolysis of cloxacillin at pH 4.0 yields an electroactive product which can be determined by polarography. Depending on the concentration of cloxacillin, one of 2 peaks was obtained: The potential ac peaks for a 4.36 mM cloxacillin solution were peak I, −0.23 V and peak II, −0.13 V. For analytical purposes, the first peak was used. A linear relationship was established for levels of cloxacillin between 2.2×10^{-5} and 2.2×10^{-3} M. Average recovery was 98.8% (SD 1.8), indicating satisfactory accuracy for the method. Individual capsule and composite assays as well as interference tests are described.

Cloxacillin is a semisynthetic β-lactam antibiotic, widely used in treating infections caused by penicillinase-producing microorganisms. Although antibiotics have traditionally been assayed by microbiological techniques (1), some chemical procedures have been developed, i.e., iodine absorption (2), ultraviolet spectrophotometry (3), and gas chromatography (4). Also, a polarographic technique has been described for this antibiotic (5).

In a recent preliminary note (6), we discussed the possibility of applying an indirect polarographic method to several antibiotics that lack an amino group in their side chains. In this work, we describe application of the method to the analysis of cloxacillin. Recovery studies, composite assays, individual capsule assays, and interference tests are described.

METHOD

Apparatus and Reagents

(a) *Polarograph.*—Operated in ac polarographic mode. Tacussel assembly, EPL-2 recorder equipped with TIPOL plug-in, GCMS pulse generator for hammer control, MPO-3 hammer, and ADAPAL unit for polarography with superimposed ac signal. Operating conditions: drop time, 1 s; potential sweep rate, 10 mV/s; frequency of superimposed signal, 20 Hz; amplitude of superimposed signal, 10 mV; phase angle, 0°.

(b) *Polarographic cell.*—Tacussel CPRA measuring cell with dropping mercury electrode, platinum wire counter electrode, and saturated calomel reference electrode (SCE).

(c) *Buffer system.*—For pH 1.0–2.0, Clark-Lubs buffer; pH 3.0–8.0, Sorensen phosphate-citrate buffer; pH 9.0–12.0, Sorensen glycine II. Hydrolyses were performed in the different buffer systems at constant ionic strength ($\mu = 1.0$).

(d) *Standard solutions.*—Accurately weigh 2.5–25 mg cloxacillin standard (100% chromatographically pure, 98.3% activity; Laboratorio Chile S.A., Santiago, Chile), dissolve, and dilute to 25 mL with pH 4.0 phosphate-citrate buffer. Heat standards 45 min in 90 ± 0.5°C constant temperature bath, and proceed with polarographic determination.

Preparation of Synthetic Samples

Prepare excipient powder for recovery studies according to manufacturer's batch formulas for 250 mg capsules. Add 250 mg cloxacillin standard, dissolve mixture, and dilute to 500 mL with pH 4.0 phosphate-citrate buffer. Then follow procedure for standard solutions.

Preparation of Composite of Samples

Weigh and finely powder contents of 25 cloxacillin capsules. Accurately weigh and transfer portion of powder equivalent to 250 mg cloxacillin into 100 mL volumetric flask and dilute to volume with pH 4.0 phosphate-citrate buffer; then dilute a portion of the solution 5-fold with pH 4.0 buffer and proceed with hydrolysis and polarography.

Preparation of Interference Samples

Prepare samples with 1.0 mL plasma containing 12.5 mg cloxacillin and transfer to 25 mL volumetric flask. Add different interfering substances, dissolve, and dilute to volume with pH 4.0 buffer.

Polarography

Transfer 15 mL of each solution to dry polarographic cell and degas by bubbling nitrogen

Received December 8, 1981. Accepted March 23, 1982.

0004-5756/82/6506-1403-04$01.00

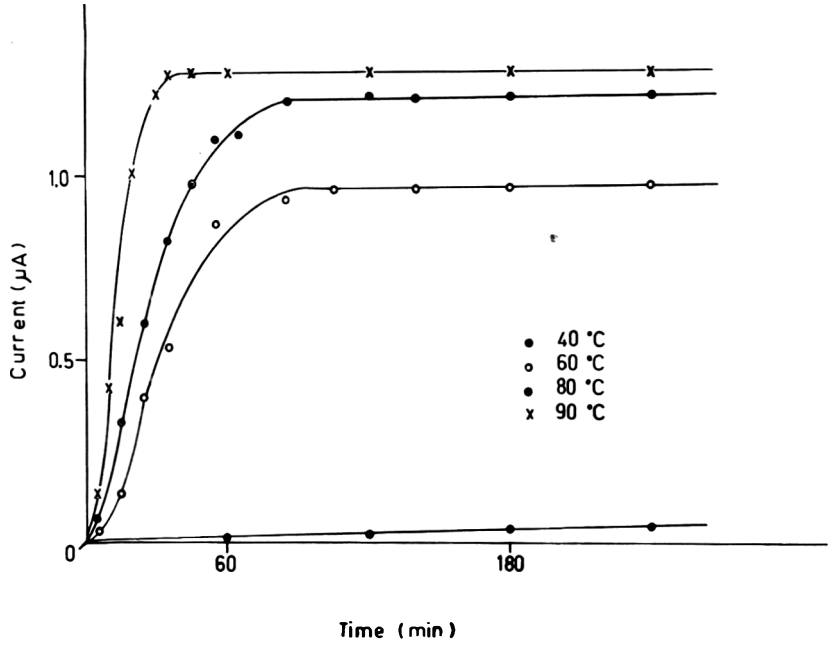


Figure 1. Maximum peak current for hydrolyzed cloxacillin at pH 4.0 and different temperatures. Initial concentration 1.0 mg cloxacillin/mL.

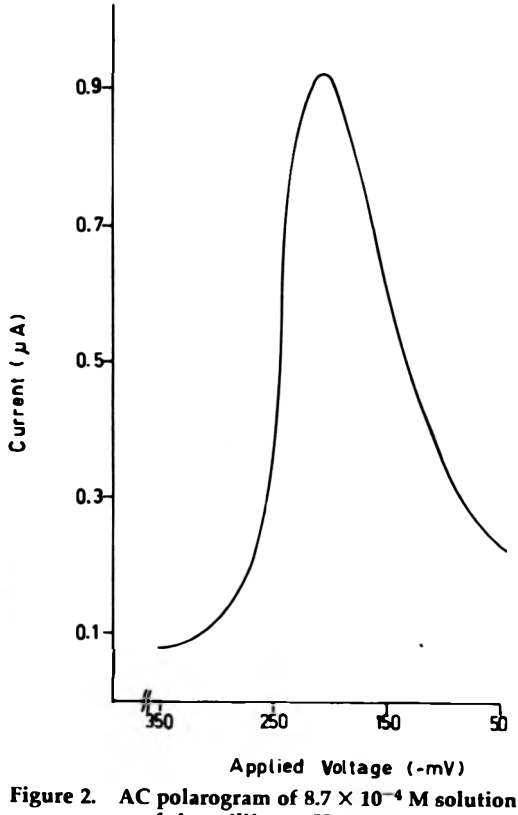


Figure 2. AC polarogram of 8.7×10^{-4} M solution of cloxacillin at pH = 4.0.

through the solution 10 min. Scan each solution at least twice between −350 and 0.0 mV. Calculate mg cloxacillin in sample solution from prepared standard calibration curve.

Measure potentials with respect to saturated calomel electrode.

Results and Discussion

Cloxacillin does not have inherent polarographic activity; therefore, a derivatization procedure must be used to achieve an indirect method of analysis. To obtain a polarographically useful response, we studied different hydrolysis conditions. Hydrolysis was carried out over the full pH range. The amount of electroactive degradation product was greatest at pH 4.0. Figure 1 shows the effect of temperature on the hydrolysis of cloxacillin. Hydrolysis conditions selected were pH 4.0, 90°C, and 45 min.

In a previous dc polarographic assay for flucloxacillin (7), we selected different hydrolysis conditions (pH 4.9, 90°C, and 180 min) because at pH 4.0 the dc polarographic anodic wave of flucloxacillin was not resolved, and the heating time had to be significantly longer than for cloxacillin to complete hydrolysis.

The electroactive oxidation product obtained under optimal hydrolysis conditions showed a

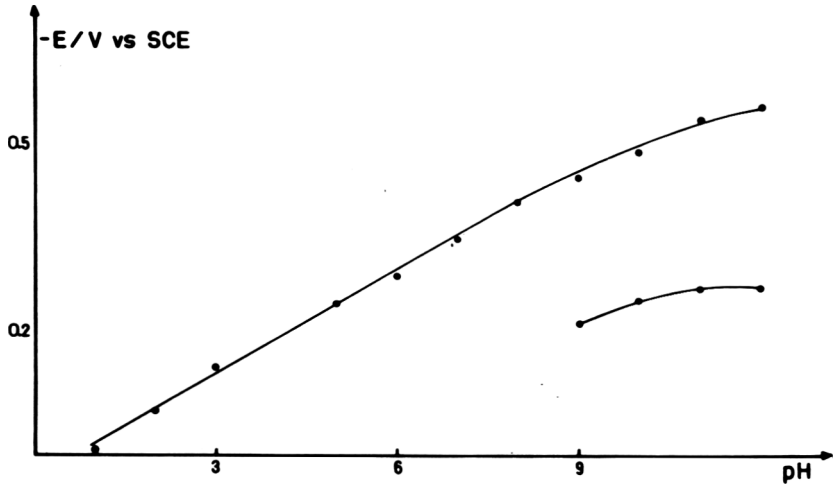


Figure 3. Plot of E_p vs pH for hydrolyzed cloxacillin at pH 4.0, 90°C, and 45 min.

peak potential of −0.20 V at pH 4.0 for an 8.7 × 10^{-4} M cloxacillin solution (Figure 2). This peak is strongly pH-dependent (Figure 3). A second peak appears between pH 9.0 and 12. The plot of E_p vs pH gives a straight line between pH 1 and 8. This line fits the equation

$$E_p\,(mV) = -55.18\ pH + 38.27 \text{ (correlation coefficient 0.998)}$$

Although the identity of the electroactive product is unknown, this pH-dependent behavior suggests a compound containing an —SH group (8). This assumption was confirmed by blocking the ac signal with *p*-hydroxy mercury benzoate.

The $\Delta E_p/\Delta pH$ value of −55.18 indicates a one electron, one proton process, which would be illustrated by the following equation:

$$Hg + R—SH \rightleftharpoons Hg(SR) + H^+ e^-$$

The electrode process is controlled by the diffusion of R—SH to the mercury drop surface. This statement is supported by the low temperature coefficient (1.10%/°C between 5 and 50°C) and the linearity observed by dc polarography between the diffusion current and the square root of the height of the mercury column (corrected for back pressure). This equation is:

$$i_p(\mu amp) = (H^{1/2}_{corr.}\ (cm) \times 0.557) - 0.825$$

The correlation coefficient for 7 values was 0.996 for H between 30 and 70 cm. The electroactive degradation product peak current exhibits a linear relationship with concentration levels of cloxacillin between 2.20 × 10^{-5} and 2.20 × 10^{-3} M. A second peak started to appear at about 3.20 × 10^{-3} M cloxacillin. The effect of concentration

Table 1. Effect of concentration on ac polarography of cloxacillin solutions [a]

Concn (M × 10^3)	No. of waves	$-E_p$, V	i_p, μamp
0.22	1	0.22	0.21
0.44	1	0.22	0.45
1.10	1	0.22	0.87
2.00	1	0.22	1.21
2.20	1	0.22	1.32
4.36	2	0.23	2.38
		0.13	0.66
6.55	2	0.23	2.34
		0.12	0.50
8.72	2	0.23	2.28
		0.10	0.34

[a] Cloxacillin solutions were hydrolyzed 45 min at 90°C in pH 4.0 buffer.

Table 2. Recovery of cloxacillin from synthetic capsules [a]

	Found, mg	Rec., %
	242.5	97.0
	253.8	101.5
	247.5	99.0
	242.5	97.0
	253.8	101.5
	245.0	98.0
	245.8	98.3
	244.5	97.8
Av.	246.9	98.8
SD	4.5	1.8
CV, %	1.8	1.8

[a] Each synthetic mix represents a commercial formulation containing 250 mg cloxacillin.

Table 3. Multiple analyses of cloxacillin samples, mg found

	Capsules[a]	Composite[b]
	263.7	251.5
	248.5	247.8
	246.7	246.0
	238.9	247.8
	253.3	257.0
	254.0	245.0
	264.3	248.3
Av	252.8	249.1
SD	9.1	4.1
CV, %	3.6	1.6

[a] Cloxacillin capsules (Recalcine Laboratories SA, Santiago, Chile), declared amount 250 mg/capsule. Individual capsule assay is expressed as mg/single capsule weight.

[b] 325 mg sample of powder containing cloxacillin plus excipients equivalent to average capsule weight of 25 capsules.

Table 4. Effect on peak current and potential of cloxacillin in cloxacillin-plasma samples[a] containing endogenous and exogenous substances

Interference added	i_p, μamp	$-E_p$, V
None		
Drug-free plasma	0.73	0.22
Diazepam (35.11 μmol/L)	0.78	0.22
Phenobarbital (193.77μmol/L)	0.78	0.21
Codeine (16.7 μmol/L)	0.77	0.22
Paracetamol (132.31 μmol/L)	0.72	0.21
Theophylline (83.25 μmol/L)	0.77	0.22
Bilirubin (855.21 μmol/L)	0.80	0.22
Urea (15.65 mmol/L)	0.77	0.22

[a] All cloxacillin–plasma samples contained 0.5 mg cloxacillin/mL.

on number of peaks is summarized in Table 1. Peak height increases with concentration up to about 10^{-3} M and then decreases slightly. The potential of the second peak was dependent on concentration.

Similar behavior has been reported for penicillamine (9); however, the electroactive degradation product obtained in this hydrolysis is not penicillamine. In this study, for cloxacillin (initial concentration, 0.5 mg/mL) hydrolyzed 45 min at 90°C in pH 4.0 buffer, peak current of the degradation product at room temperature and pH 4.0 was 0.71, 0.70, 0.69, 0.68, 0.66, 0.61, and 0.14 μamp at 0.0, 0.5, 1.0, 3.0, 6.0, 22.0, and 150.0 h, respectively. This decrease is not important at short times; however, we recommend running polarograms within the first 20 min, to obtain good reproducibility.

For analytical purposes, we used the first peak. To determine cloxacillin concentration, we applied the calibration curve method. The equation is:

$$i_p(\mu\text{amp}) = 1.17\ C\ (\text{mg/mL}) + 0.2$$

The correlation coefficient for 9 values was 0.987 between 0.1 and 1.0 mg cloxacillin/mL.

Data are shown in Table 2 for the recovery of cloxacillin from synthetic capsules. The average recovery of 98.8% (SD 1.8) indicates satisfactory accuracy for the developed method.

Results of individual capsule and composite assays are shown in Table 3. The higher SD value obtained for the individual capsule assay is probably due to differences in the weight of individual capsules.

We studied possible interferences in the assay from endogenous substances and from some drugs commonly administered together with cloxacillin. No significant interference in this assay was found for any substance listed in Table 4. Therefore, the developed method may be used for estimating cloxacillin in the presence of such compounds and may be especially useful in control of uremic patients.

Compared with the previously described polarographic assay (5), the present method is faster and preparation of samples is easier.

Acknowledgments

This work was supported by Servicio de Desarrollo Científico, Creación Artística y de Cooperación International (Q-1132/8113). We thank P. Fuhrman for his technical assistance in purifying a reagent.

References

(1) Bennet, J. V., Brodie, J. L., Benner, E. J., & Kirby, W. M. M. (1966) *Appl. Microbiol.* **14**, 170–174

(2) Hou, J. P., & Poule, J. W. (1969) *J. Pharm. Sci.* **58**, 1510–1514

(3) Bundgaard, H., & Ilver, K. (1972) *J. Pharm. Pharmacol.* **24**, 790–793

(4) Kawai, S., & Hashiba, S. (1964) *Bunseki Kagaku* **13**, 1223–1226

(5) Benner, E. J. (1970) presented at the 10th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, Oct. 18–21

(6) Squella, J. A., & Núñez-Vergara, L. J. (1981) *J. Electroanal. Chem.* **130**, 361–366

(7) Squella, J. A., Silva, M. M., & Núñez-Vergara, L. J. (1981) *Talanta* **28**, 855–856

(8) Jemal, M., & Knevel, A. (1978) *Anal. Chim. Acta* **96**, 143–147

(9) Jemal, M., & Knevel, A. (1979) *J. Electroanal. Chem.* **95**, 201–210

Quantitative Estimates of Beta-Lactam Residues in Raw Milk Around a Reference Standard: Collaborative Study

ROY E. GINN, RONALD A. CASE,[1] VERNAL S. PACKARD,[2] and SITA R. TATINI[2]
Dairy Quality Control Institute, Inc., 2353 N Rice St, St. Paul, MN 55113

Collaborators: E. Bone; M. Brizendine; A. Curme; P. R. Danilowier; R. Fuqua; H. H. Grant; J. Heady; H. Henrickson; W. N. Kelley; J. E. Leslie; D. E. Schneider; R. R. Williams

A collaborative study was conducted to determine the reliability of a *Bacillus stearothermophilus* disc assay method for differentiating various concentrations of penicillin in raw milk. Participating laboratories tested 10 different samples (including one negative) in blind duplicate. Triplicate standards were alternated with triplicate unknowns around the periphery of each of 5 different plates. Zone diameters were measured and the difference in zone size of pairs of adjacent standard and unknown samples were analyzed by a paired *t*-test. Penicillin concentrations 0.003 IU/mL different from the reference concentrations were consistently distinguishable at a 95% confidence level. Such discriminatory power was determined to be possible with as few as 3 plates (9 replicates) per unknown. The method has been adopted official first action.

In anticipation of a proposed FDA tolerance for antibiotics in milk and dairy products, The National Conference on Interstate Milk Shipments (NCIMS) commissioned a study to investigate a rapid, simple quantitative test for antibiotics in milk and dairy products. In recent years, a number of investigations have been undertaken to perfect and refine qualitative methods using *Bacillus stearothermophilus* as the test organism (1–5). This work eventually culminated in a commercial disc assay test, referred to as the Difco disc assay method. This procedure is rapid, requiring only 2¾ h of incubation, and is sensitive to penicillin at levels as low as 0.002 unit/mL milk (6). In the work reported herein, certain refinements were introduced into this disc assay procedure, a method was devised for differentiating unknown concentrations of penicillin from a given reference concentration, and a collaborative study was undertaken to determine the reliability of this method.

Penicillin Residues in Milk

Quantitative *Bacillus stearothermophilus* Disc Method

Official First Action

(Applicable to levels ≥0.016 IU penicillin G/mL)

16.C12 ***Apparatus and Reagents***

(a) *Microliter pipetter.*—90 μL, with disposable tips (Eppendorf Model, Curtin Matheson Scientific, Inc., PO Box 1546, Houston, TX 77001).

(b) *Vernier calipers.*—Readable to 0.1 mm (Curtin Matheson Scientific, Inc.).

(c) *Petri dishes.*—Flat type (Falcon Series, Curtin Matheson Scientific, Inc.).

(d) *Filter discs.*—Non-sterile, ½ in. diam., round 740E (Schleicher and Schuell, Inc., 543 Washington St, Keene, NH 03431).

(e) *Penicillinase discs.*—Round, ½ in. diam. (Difco Laboratories).

(f) *Incubator.*—Capable of maintaining temp. 64 ± 2°.

(g) *Assay medium.*—Antibiotic Medium 4 (Difco Laboratories), **42.196(b)**.

(h) *Spore suspension.*—Stdzd *B. stearothermophilus* spore suspension, ATCC No. 10149 (Difco Laboratories).

(i) *Phosphate buffer.*—Dissolve 8 g anhyd. KH_2PO_4 and 2 g anhyd. K_2HPO_4 in 1 L distd or deionized H_2O. Adjust to pH 6.0 if necessary.

(j) *Std soln.*—1000 IU USP Na penicillin G Ref. Std/mL phosphate buffer. This stock soln may be frozen for later use. For assay, dil. first to 1.0 IU/mL in phosphate buffer and then prep. 0.016

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.* 65, 389 (1982).

Received August 10, 1981. Accepted May 15, 1982.

[1] Kraft, Inc., Kraft Court, Glenview, IL 60025.

[2] Department of Food Science and Nutrition, University of Minnesota, 1354 Eckles Ave, St. Paul, MN 55108.

0004-5756/82/6506-1407-06$01.00

IU/mL ref. std milk by dilg aliquot with previously tested penicillin-free milk.

16.C13 *Preparation of Seeded Medium and Culture Plates*

Prep. sterile Antibiotic Medium 4 in flasks in 100 mL lots. Add 1 mL ampule of *B. stearothermophilus* spore suspension to each 100 mL lot previously tempered to 65°. Preferably, seed fresh each day of testing. Work only on absolutely flat surface. Using warm 10 mL pipet, transfer 6 mL seeded medium to center of petri dish. Cover dish and swirl gently to cover entire bottom surface. Let medium solidify at room temp.

16.C14 *Procedure*

Let stock std and samples thaw (if frozen) at room temp. Prep. 0.016 IU/mL ref. std milk, (j). Heat ref. std milk and samples to 82° for 2 min. Cool in ice-H_2O bath.

Using clean, dry forceps, place 6 filter discs evenly spaced around periphery of culture plate, ca 1 cm from outer edge. Touch each disc gently to surface of medium with tips of forceps. Using sep. forceps, place penicillinase disc in center of plate.

Within 45–60 s after disc is placed on culture plate, add 90 μL ref. std or sample. Alternate ref. std with samples on each plate, adding sample to penicillinase disc. Hold pipetter over center and 3–5 mm above disc when adding sample. Eject sample in even flow; avoid splash. Use 1 disposable tip for triplicate ref. stds and 1 for triplicate samples. Prep. replicate plates: 3 plates (total of 9 replicate ref. stds and 9 replicate samples) provide satisfactory discrimination on quantitative basis.

Invert plates and place immediately in 64 ± 2° incubator. Incubate 2 h and 45 min. Remove plates in sets as prepd, and place on laboratory bench at room temp. while reading.

16.C15 *Measurement of Zone Diameter*

Using vernier calipers, measure zone diam. of ref. std and sample to nearest 0.1 mm. Include both disc and zone of inhibition in this measurement. If present, measure any zone found around penicillinase disc, record as "other inhibitor," and proceed no further with test. If no zone of inhibition is found around penicillinase disc, and if zone diams of sample are larger than those of ref. std, proceed with calcns.

16.C16 *Calculations*

Det. validity of difference between ref. std and sample zone diam. by paired-*t* analysis using paired differences that occur between each ref. std and clockwise adjacent unknown. Each plate thereby yields 3 differences for use in paired-*t* analysis. Calc. *t*-value, $t = \sqrt{n} \times \bar{d}/S_d$, where n = number of observations, $\bar{d}$ = mean of differences, and S_d = std dev. of differences =

$$\sqrt{\frac{\Sigma(d_i - \bar{d})^2}{n-1}} = \sqrt{\frac{\Sigma d_i^2 - \left(\frac{(d_i)^2}{n}\right)}{n-1}}$$

Simplified formula for calcg *t* for 9 replicates (3 plates) is as follows:

$$t = \frac{2.828 \times D_1}{\sqrt{9 \times D_2 - (D_1)^2}}$$

where D_1 = sum of differences of 9 replicate detns; and D_2 = sum of squares of differences of 9 replicate detns.

A *t*-value >1.860 indicates with 95% confidence that sample contains ≥0.016 IU penicillin/mL.

Collaborative Study

A negative control and 9 different concentrations of penicillin were used in this study, as follows: 0.012, 0.014, 0.015, 0.016, 0.017, 0.018, 0.019, 0.020, and 0.022 IU/mL milk. All 10 samples were split and each laboratory received 20 samples in blind duplicate, thus 18 samples contained measurable levels of penicillin. Reference levels of penicillin were also prepared in milk and each laboratory received sufficient amounts to perform all tests. The reference level was set at 0.016 IU penicillin/mL. However, the zone diameters, in preliminary analysis, were averaged and ranked in order by size vs concentration. At this point, it was discovered that the reference concentration appeared to more nearly reflect a level of 0.015 than 0.016 unit of penicillin/mL. Further analysis revealed that the reference concentration did in fact differ from 0.016 to a statistically significant degree. Given these facts, a concentration of 0.015 unit/mL (the more conservative figure) was taken as the reference point for analyzing results.

Results and Discussion

Ten laboratories participated in the study. Each laboratory was asked to prepare 5 replicate plates (15 pairs of determinations) per unknown (Table 1). Taking precision to mean the square root of the average variance observed among the 18 samples that contained penicillin, laboratory precision ranged from 0.26 to 0.91 for 9 of the 10 laboratories (see Table 2). The average precision

Table 1. Summary of collaborative data for quantitative estimates of β-lactam residues in raw milk around a reference standard [a]

Lab.	Concn. of unknown	Av. zone unknown	Av. zone std	Av. diff.	SD of diff.	Av. *t*-value, 5-plate groups	Av. *t*-value, 3-plate groups
1	0.012	17.96	18.33	−0.37	0.526	−2.702	−2.288
	0.014	17.28	17.44	−0.16	0.334	−1.881	−1.442
	0.015	18.15	18.21	−0.06	0.428	−0.294	−0.168
	0.016	18.17	17.83	0.34	0.413	3.173	2.564
	0.017	18.44	18.27	0.18	0.388	1.733	1.390
	0.018	18.58	17.89	0.69	0.576	4.643	3.772
	0.019	18.60	17.94	0.66	0.446	6.123	4.953
	0.020	18.54	17.66	0.88	0.507	6.685	5.640
	0.022	18.97	17.60	1.37	0.434	13.254	10.725
2	0.012[b]	17.85	18.18	−0.33	0.896	−1.441	−1.107
	0.014	17.88	18.11	−0.24	0.518	−1.944	−1.546
	0.015	18.06	18.20	−0.14	0.751	−0.684	−0.569
	0.016	18.49	18.36	0.13	0.437	0.984	1.015
	0.017	18.24	18.04	0.20	0.485	1.738	1.403
	0.018	19.02	18.51	0.51	0.904	2.700	2.829
	0.019	18.98	18.10	0.88	0.593	5.675	4.562
	0.020	19.13	18.31	0.82	0.780	4.557	3.540
	0.022	19.37	17.78	1.59	0.913	7.499	7.216
3	0.012	16.45	17.03	−0.58	0.533	−4.157	−3.450
	0.014	17.07	17.26	−0.20	0.842	−1.389	−1.216
	0.015	17.53	17.43	0.09	0.507	0.926	−1.350
	0.016	17.63	17.36	0.27	0.825	1.149	0.715
	0.017	17.98	16.91	1.07	1.107	4.285	0.928
	0.018	18.24	17.65	0.59	0.940	2.419	3.555
	0.019	18.05	17.18	0.87	0.862	5.421	1.883
	0.020	18.46	17.55	0.91	1.030	4.089	4.429
	0.022	18.82	17.44	1.38	0.636	8.790	3.182
4	0.012	17.16	17.91	−0.74	0.256	−11.352	6.939
	0.014	17.54	17.75	−0.21	0.302	−2.880	−9.401
	0.015	17.63	17.66	−0.04	0.303	−0.473	−2.271
	0.016	17.57	17.30	0.27	0.311	3.294	−0.331
	0.017	18.08	17.62	0.46	0.239	7.993	2.549
	0.018	18.45	17.80	0.65	0.290	10.214	6.378
	0.019	18.70	17.98	0.72	0.223	12.550	8.404
	0.020	19.09	18.11	0.97	0.182	21.087	10.802
	0.022	19.09	17.70	1.39	0.200	31.355	16.784
5	0.012	16.72	17.55	−0.83	1.104	−2.995	25.314
	0.014	17.02	17.30	−0.29	0.297	−3.776	−4.777
	0.015	17.54	17.73	−0.19	0.319	−2.244	−2.988
	0.016	17.77	17.69	0.08	0.331	1.198	−1.775
	0.017	18.45	17.98	0.46	0.339	5.653	0.873
	0.018	18.32	17.85	0.47	0.304	6.021	4.823
	0.019	19.20	18.53	0.67	0.366	10.043	5.297
	0.020	18.72	17.89	0.83	0.961	6.800	8.552
	0.022	19.21	18.13	1.08	0.422	13.839	6.385
6	0.012	17.47	18.08	−0.61	0.306	−8.226	11.099
	0.014	17.97	18.21	−0.23	0.453	−2.147	−6.458
	0.015	18.27	18.34	−0.07	0.317	−0.876	−1.801
	0.016	18.58	18.38	0.20	0.325	3.237	−0.679
	0.017	18.33	18.09	0.24	0.323	2.877	2.674
	0.018	18.93	18.22	0.70	0.361	7.824	2.267
	0.019	18.98	18.31	0.67	0.338	8.033	6.671
	0.020	18.97	18.07	0.90	0.312	13.132	6.568
	0.022	19.07	17.85	1.23	0.393	11.923	10.591
7	0.012	17.64	18.40	−0.76	0.555	−5.593	10.979
	0.014	17.27	17.39	−0.12	0.505	−0.748	−4.616
	0.015	17.98	18.07	−0.09	0.606	−0.556	−0.571
	0.016	18.03	17.67	0.36	0.655	2.160	−0.398
	0.017	17.98	17.48	0.50	0.551	3.840	1.676
	0.018	19.21	18.51	0.69	0.598	4.416	3.160
	0.019	18.64	17.83	0.81	0.657	4.943	3.473

Table 1. (continued)

	0.020	18.78	17.85	0.93	0.468	8.020	3.839
	0.022	18.96	17.53	1.43	0.504	12.710	6.311
8	0.012	17.84	18.15	−0.31	0.505	−2.355	10.188
	0.014	17.66	17.99	−0.32	0.334	−4.623	−2.407
	0.015	18.21	18.11	0.10	0.322	0.562	−4.002
	0.016	18.71	18.65	0.06	0.400	0.837	0.158
	0.017	18.45	18.33	0.12	0.328	1.858	0.293
	0.018	18.89	18.47	0.42	0.581	3.463	1.484
	0.019	19.30	18.47	0.83	0.551	6.111	2.751
	0.020	19.10	18.30	0.80	0.359	8.946	5.463
	0.022	19.61	18.55	1.07	0.243	16.968	8.534
9	0.012	19.37	19.97	−0.60	1.597	−1.491	13.431
	0.014	19.30	20.06	−0.77	1.312	−2.853	−1.293
	0.015	20.19	20.29	−0.10	1.116	−0.301	−2.248
	0.016	19.79	19.01	0.78	0.954	3.191	−0.271
	0.017	20.41	19.90	0.51	2.379	0.897	2.515
	0.018	19.81	18.86	0.95	1.189	3.648	0.794
	0.019	20.20	19.39	0.80	1.036	3.224	2.902
	0.020	20.86	19.37	1.49	1.364	4.307	2.608
	0.022	21.73	20.48	1.25	1.909	2.665	3.388
10	0.012	17.14	17.87	−0.72	0.399	−7.054	2.278
	0.014	17.53	17.82	−0.29	0.406	−3.144	−5.640
	0.015	17.90	17.99	−0.09	0.482	−0.711	−2.469
	0.016	18.06	17.95	0.11	0.383	1.268	−0.580
	0.017	17.94	17.82	0.11	0.908	1.038	0.984
	0.018	18.57	18.07	0.50	0.409	5.042	1.018
	0.019	18.58	17.95	0.63	0.371	6.848	3.958
	0.020	19.06	18.24	0.81	0.463	6.748	5.576
	0.022	19.76	18.26	1.50	0.704	10.547	5.879

[a] Because 5 plates per concentration of unknown were prepared, the average *t*-value is shown both for 5 plates and 3 plates, the latter derived by testing all combinations of 3 plates within the 5-plate parameter. Number of tests at each unknown concentration = 30 except where noted.

[b] Number of tests = 15.

for these 9 laboratories was 0.45. Precision of the tenth laboratory was 1.40, more than 3 times the average for the other nine. For this reason, data from this one laboratory were excluded from further analysis. In addition, certain other laboratories either reported a laboratory accident on one sample or, in one case, one laboratory appeared to have one disc mis-located (confused) on same plates. Taking account of these problems, Table 3 indicates the number and percentage of samples at each concentration that would have been validated by a paired *t* test (at 95% confidence) as being above the reference level (0.015 unit/mL) of antibiotic in milk. Data express results for 5 plates (15 pairs of standard and unknown replicates) and 3 plates (9 pairs of standard and unknown replicates). The latter values were computed from the data obtained on 5 plates. This was done by analyzing all possible combinations of 3 pairs for each of the 5 plates, i.e., 10 combinations. Data in Table 3 also show results obtained for 9 laboratories and for 7 laboratories. This latter number of laboratories, along with the number of samples tested, still meets the statistical requirements for a collaborative study. At the same time, however, it excludes results of 2 laboratories in which some obvious problems arose, no doubt as a result of unfamiliarity with the test. None of the laboratories had had any prior experience with the specific application of the procedure used in this study. One laboratory (excluded from this latter evaluation) reported difficulty or possibility of

Table 2. Precision of collaborating laboratories in estimating concentrations of β-lactam above a reference standard

Lab.	Precision[a]
1	0.45
2	0.63
3	0.91
4	0.26
5	0.31
6	0.34
7	0.57
8	0.39
9	1.40
10	0.45

[a] Precision is defined as the square root of the average variance in the 18 analyses made by each participating laboratory.

Table 3. Collaborative results in discriminating levels of concentration of β-lactam above a reference concentration [a]

Concn, of penicillin, IU/mL	9 Labs 15 Pairs 18 Comparisons [b]		7 Labs 15 Pairs 14 Comparisons [c]		9 Labs 9 Pairs 180 Comparisons [d]		7 Labs 9 Pairs 140 Comparisons [e]	
	No.	%	No.	%	No.	%	No.	%
0.012[f]	0	0	0	0	0	0	0	0
0.014	0	0	0	0	0	0	0	0
0.015	1	6	0	0	11	6	1	0.7
0.016	10	56	8	57	76	42	71	51
0.017	14	78	11	79	111	62	81	58
0.018[g]	16	94[h]	13/13	100	147/170	86[h]	129/130	99
	16/16	100		100	146/160	91		99
0.019	18	100	14	100	178	99	140	100
0.020[i]	17/17	100	13/13	100	164/170	96	130/130	100
0.022[j]	15/15	100	11/11	100	150/150	100	110/110	100

[a] Reference level is taken as 0.015 unit of β-lactam residue in 1 mL milk.

[b] Eighteen comparisons with 9 laboratories analyzing 2 samples at each concentration of penicillin.

[c] Fourteen comparisons with 7 laboratories analyzing 2 samples at each concer tration of penicillin.

[d] These comparisons consist of 9 laboratories analyzing 2 samples at each concentration of penicillin, and for 10 different combinations of 3 data-sets.

[e] These comparisons consist of 7 laboratories analyzing 2 samples at each concentration of penicillin, and for 10 different combinations of 3 data-sets.

[f] Laboratories 1 and 2 reported a laboratory accident on one sample each at this concentration. These sample results are not included in the tabulated data.

[g] Laboratory 2 reported no zone on one reference standard. This sample result is not included in the tabulated data.

[h] Laboratory 8 appeared to mis-locate the disc on one sample. Data in the table express results with and without this sample included.

[i] Laboratory 5 reported a laboratory accident on one sample. The tabulated data exclude this sample.

[j] Laboratories 2, 5, and 6 reported one laboratory accident each on one sample at this concentration. The tabulated data exclude these samples.

mis-use of the pipetter. Amount of milk delivered to the discs might well have been inconsistent or inaccurate. In the second laboratory that was excluded, lack of precision indicated that problems occurred. Precision within both of these laboratories was significantly poorer than the other 7, thus pointing to the likelihood of some failure in performance of the test. While the 9 laboratories together averaged 0.45 in precision, the 2 excluded laboratories had precision values of 0.63 and 0.91. With more experience, all laboratories might be expected to obtain results within the level of precision and discrimination found in the 7 more proficient laboratories participating in this study.

Lastly, data in Table 3 are expressed in terms of a given number of comparisons made at each level of antibiotic tested. The column designated as 18 comparisons represents 9 laboratories analyzing 2 samples at each concentration of penicillin ($9 \times 2 = 18$). The column designated as 180 comparisons represents 9 laboratories analyzing 2 samples at each concentration of penicillin and for 10 different combinations of 3 pairs as reflected in 5 different plates; thus $9 \times 2 \times 10 = 180$. The same reasoning holds for the 7-laboratory analyses at 14 and 140 comparisons.

Results in Table 3 suggest a potential for laboratories to discriminate at a level of antibiotic 0.003 IU in excess of a reference. Nine laboratories did so with 15 replicate pairs of observations. Seven laboratories achieved 99% discrimination at this level of difference, and with only 3 plates (9 replicate pairs, 140 comparisons). At a concentration 0.004 unit above the reference, essentially all laboratories validated the difference, and with as few as 9 replicate observations, i.e., 3 plates.

The authors are of the opinion that, with a minimum of experience with the test, most laboratories would achieve discriminatory power, at 95% probability, of 0.003 IU of penicillin over and above a given reference standard, and with 3 plates per sample, i.e., 9 replicate pairs. A skilled technician in the laboratory in which the test procedure was developed readily discriminates at 0.002 unit over reference, thus giving evidence of this potential. It is recommended that the method be adopted official first action.

Acknowledgments

The authors express their gratitude to Dennis Jennings and Brenda Catardi for their assistance in statistical analysis and interpretation of results, and the following collaborators:

Ester Bone, Dept of Public Health, Springfield, IL; Michael Brizendine and Judy Heady, Missouri Division of Health, Columbia, MO; Alison Curme and Herman Henrickson, Kraft, Inc., Melrose, MN; Paul R. Danilowier, Agri Mark, Inc., Newington, VT; Ruth Fuqua, Dairymen, Inc., Lexington, KY; Henry H. Grant, Jr, Florida Dept of Agriculture, Tallahassee, FL; W. N. Kelley, South Dakota Chemical Laboratory, Vermillion, SD; James E. Leslie, Food and Drug Administration, Washington, DC; Daniel E. Schneider, Kraft, Inc., Beaver Dam, WI; Robert R. Williams, Oregon Dept of Agriculture, Salem, OR.

REFERENCES

(1) Forschner, E. (1973) *Arch. Lebensm. Hyg.* **24,** 108–114
(2) Galesloot, T. E., & Hassing, F. (1962) *Versl. Med. Inst. Zuivelondersoek* **68,** 89–95
(3) Igarshi, R. T., Baugham, R. W., Nelson, R. E., & Hartman, P. A. (1961) *J. Milk Food Technol.* **24,** 143–146
(4) Terplan, G. K., Zaadhor, K. J., Angerbach, H., & Skarlackidou, M. (1973) *Arch. Lebensm. Hyg.* **24,** 90–92
(5) Ouderkirk, L. A. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1116–1118
(6) Ginn, R. E., Tatini, S., & Packard, V. S. (1978) *J. Food Prot.* **41,** 361–363

Modified Nuclear Magnetic Resonance Assay of Gentamicins

NICHOLAS P. REUTER, A. CAROL HANEKE, EVELYN LEWIS, THOMAS G. ALEXANDER, EUGENE MAZZOLA,[1] and ADORJAN ASZALOS
Food and Drug Administration, National Center for Antibiotics, Washington, DC 20204

An NMR spectrometric method was designed to assay the fractional "C" composition of gentamicin preparations. The gentamicin C_1 and C_2 contents of the samples are calculated from the *N*-methyl and *C*-methyl integration ratios of the corresponding functional groups of the gentamicin structures. Gentamicin C_{1a} content is obtained by subtraction of ($C_1 + C_2$)% from 100%. This new NMR assay is applicable to 90 MHz NMR instruments and is more accurate and less time consuming than the presently used official assay described in the Code of Federal Regulations.

Gentamicins (1) are a family of related aminoglycoside antibiotics used extensively against various Gram-positive and Gram-negative microbial infections. The structures of the 3 main "C" components in commercial preparations of gentamicin, C_1, C_2, and C_{1a}, are shown in Figure 1. Neither the relative antimicrobial activity nor, more important, the relative toxicity of the individual members of the gentamicin family has been clearly established. Therefore, it is important that the fractional composition of the commercially available gentamicin be maintained within the limits established by the clinically tested materials.

Several testing systems are available to determine this composition. Some of the existing test systems rely on thin layer chromatography, paper chromatography, or ion-exchange column chromatography (2–5). For example, the Code of Federal Regulations (CFR) (6) describes a test based on paper chromatographic separation of the 3 main gentamicin components followed by microbiological assay of the individual members. Because these methods do not possess the desired combination of speed, specificity, and precision, other types of analytical procedures were investigated. An analytical system based on HPLC separation with post-column derivatization and fluorescence detection proved to have difficult instrumentation problems (7). Another method employing the fast nuclear magnetic resonance (NMR) technique was developed recently (8) and is based on peak height ratio estimation of *C*- and *N*-methyl groups in the NMR spectrum. An estimation of the composition of the different gentamicins in the analyzed sample can be computed by using empirical formulas. The empirical formulas were derived for use with spectra from a 60 MHz NMR instrument and were not applicable to the 90 MHz instrument available to this laboratory. Our modified method demonstrates that accurate values can be obtained by using *C*- and *N*-methyl ratios obtained from electronic integration instead of from peak height measurements. Details of this modified assay are described below.

METHODS

Materials

Gentamicin sulfate.—Samples were obtained from the Food and Drug Administration's Certification Submissions and New Drug Applications (NDA) for Marketing program. The purified gentamicin sulfate components C_1, C_2, and C_{1a}, are supplied by Schering Co. All samples were dried 3 h at 105°C before analysis.

Determination

Dissolve individual samples in deuterium oxide to a final concentration of 20% (w/v). Add 0.05% (w/v) Na 3-trimethylsilylpropanesulfonate (TMPS) zero frequency reference standard (Eastman Organic Chemicals).

Record individual spectra on a Varian 90 MHz EM-390 NMR spectrometer. Adjust phase with reference to deuterium oxide (δ5 ppm) and TMPS (δ0 ppm) signals. Manipulate spectrum amplitude with several scans to maximize the *N*-methyl peak which occurs at δ2.95 ppm. Use sweep width of 5 ppm and sweep time of 5 min.

Perform integration sweeps at fast (1 min) scan time, with reduced spectrum amplitude setting. Adjust integration balance control to assure relatively steady baseline. Record 10 integrations with each sample for both *N*-methyl (δ2.95, 2.75 ppm) and *C*-methyl (δ1.35, 1.25 ppm) shifts.

Obtain *N*-methyl ratio directly from integration of signals at δ2.95 and δ2.75 ppm. This ratio determines the percentage of the C_1 component in the sample.

Determine percentage of $C_1 + C_2$ components

[1] Division of Chemistry and Physics.

Received February 9, 1982. Accepted May 10, 1982.

0004-5756/82/6506-1413-04$01.00

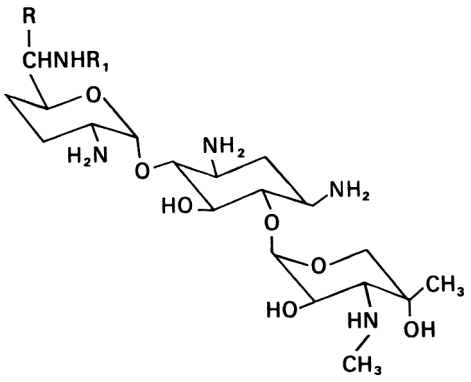


Figure 1. Structures of gentamicin components C_1, C_{1a}, and C_2. C_1: $R = R_1 = CH_3$. C_2: $R = CH_3$; $R_1 = H$. C_{1a}: $R = R_1 = H$.

in samples from spectral data with the use of an empirical equation. Read the δ1.35 ppm signal from the *C*-methyl group present in all the components as direct integral from initial baseline to first break. Because of the incomplete resolution of the methyl doublet generated by C_1 and C_2 components, it is necessary to measure the δ1.25 ppm integration in the following manner: Determine integration value by measuring observed "inflection point" produced by incomplete resolution to "arc bisecting point" culminating δ1.25 ppm integration. Determine "inflection point" precisely by drawing tangents along integral slopes and noting where tangents intersect. Quantitative value for the δ1.25 ppm integration is the distance between "arc bisecting point" and "inflection point." (See examples in Figure 2.)

Calculate combined C_1 and C_2 content of each sample by an algebraic treatment of the *C*-methyl ratio:

$$\%C_1 + C_2[a - 1.08 \times 10^{-1}]/1.5 \times 10^{-3} \quad (1)$$

where a is the *C*-methyl ratio. Subtract percentage of C_1 and C_2 from 100% to determine C_{1a} content.

The compositions of the samples were also determined by NMR peak height measurements. For these measurements the ratios were determined from the height of the respective peaks from the baseline and then subjected to the mathematical treatment as described by Calam et al. (8).

Results and Discussion

It was observed recently that the analyses of gentamicin bulk preparations as reported by a manufacturer differ greatly from those reported by FDA laboratories. Currently used analytical

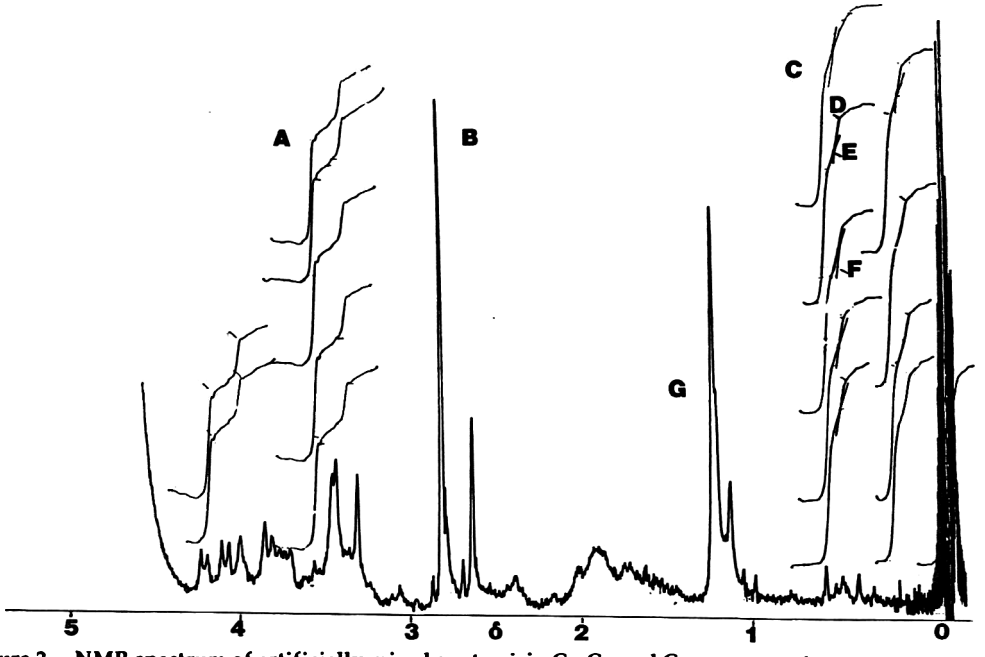


Figure 2. NMR spectrum of artificially mixed gentamicin C_1, C_2, and C_{1a} components.
Integration curves were obtained and used to calculate individual gentamicin components as described in method. A, *N*-methyl integrations; B, *N*-methyl peaks; C, *C*-methyl integration; D, arc bisecting point; E, inflection point; F, tangents; G, *C*-methyl peaks.

Table 1. Comparison of analytical data of different gentamicin preparations as performed by official paper chromatographic method (6) and by NMR spectrometry

Sample	Composition by manuf.[a]			Composition by FDA[a]			SD of indiv. complete assay	Composition by NMR[b]			SD of C_1 detn	SD of C_2 detn	Composition by NMR[c]
	C_1	C_2	C_{1a}	C_1	C_2	C_{1a}		C_1	C_2	C_{1a}			C_2
GN 11907 R	30.4	44.3	21.7	33.7	44.6	21.7	—	32.0	42.6	25.4	1.2	4.5	75.0
GN 11908 R	36.7	43.0	20.3	34.1	46.7	19.2	—	41.0	40.9	18.1	1.5	4.2	59.0
GN 11908 R	34.7	44.2	22.1	32.2	47.4	20.3	—	32.5	43.5	24.0	1.8	4.0	67.5
S 4248	37.2	45.5	17.3	33.0	49.2	17.6	1.4	39.4	42.5	18.1	0.5	3.2	—
S 4249	36.9	41.4	21.7	31.4	51.7	16.8	1.7	33.0	38.5	28.5	1.5	3.8	—
S 4250	35.3	41.7	23.0	28.7	45.9	25.3	2.7	30.5	39.5	30.0	1.0	3.5	—
Artificial mixture[d]				34.4	38.7	26.8	4.5	39.3	40.8	19.8	0.8	3.8	7.2

[a] Analysis by paper chromatography according to CFR (6).
[b] NMR technique described in this paper.
[c] NMR technique described in the literature (9).
[d] 40% C_1, 40% C_2, and 20% C_{1a}.

techniques, which are based on a paper chromatographic separation of the usually cofermented components of gentamicin C_1, C_2, and C_{1a} (9), apparently cannot separate these components present in bulk samples from this manufacturer. To circumvent this problem, the limiting test based on NMR spectrometry and described in the *British Pharmacopoeia* (10) was investigated. Briefly, the percentage composition of the C_1 component of a gentamicin preparation is calculated from the *N*-methyl peak height ratios of the NMR spectrum. The percentage composition of the C_2 component is obtained from the *C*-methyl peak height ratio measurements and the use of empirical formulas. This method is based on the fact that of the 3 main gentamicins, components C_1, C_2, and C_{1a}, only component C_1 has 2 *N*-methyl groups and only C_1 and C_2 have 2 C-methyl groups. The third main component, C_{1a}, has only one of each kind of the methyl groups, as shown in Figure 1. The percent composition of the C_{1a} component can be obtained by subtracting the values of C_1 and C_2 from 100%.

Acceptable spectra could be obtained with a Varian 90 MHz EM-390 NMR instrument and the *N*-methyl peak height ratio measurement yielded values close to those obtained by the official method. However, the percentage values obtained for the C_2 component were high, as shown in Table 1. Similarly, a high value of C_2% was obtained when an artificial mixture of the C_1, C_2, and C_{1a} components was subjected to this type of analysis. It was apparent that the utilization of the 90 MHz NMR precluded the use of the empirical formula derived for the 60 MHz instrument. The stronger field associated with the 90 MHz NMR decreases distortion and amplifies the δ1.25 ppm signal, resulting in overestimation of the C_2%. Because of the difficulties encountered with the use of peak height ratios, estimation of percent composition by NMR integration of the peaks was attempted. Acceptable integration curves were obtained for the *N*-methyl peaks and the ratio of those curves directly yielded correct C_1% composition. The standard deviation of these estimates with the artificial sample and with the commercial samples was about 1%.

Integration of the *C*-methyl peaks yielded somewhat less readable integration curves but average values obtained from about 6 integration curves yielded correct C_2% composition, as calculated by Formula 1. This formula is a modification of that used in the *British Pharmacopoeia*, where "*a*" is the measured C-methyl integration ratio (as described in Materials and Methods) and C_1 and C_2 are percent composition of these gentamicins in the sample. The standard deviations of the C_2 measurements are around 4%, as indicated in Table 1. The values obtained for C_2% composition for the artificial mixture and for the certification samples analyzed by the paper chromatographic method are in good agreement with the theoretical or official analytical values. These values are tabulated in Table 1. The analytical values obtained by FDA for samples submitted through the NDA program differ considerably from those obtained by the manufacturers. The values obtained by the NMR technique are closer to those of the manufacturers. Impurities interfering with the paper chroma-

tographic separation of the individual gentamicin components appear not to influence the NMR assay. For illustrative purposes, the significant parts of the NMR spectrum of the artificial mixture are shown in Figure 2.

Our preference for the integration method originates from the fact that the simple peak height measurements recommended by the *British Pharmacopoeia* yielded unacceptably high C_2% values on our Varian 90 MHz instrument. The reason for this discrepancy has been previously discussed. On the other hand the ratio of integration values provided accurate and precise estimation of the C_1 component and accurate but somewhat less precise estimation of the C_2 and C_{1a} components (values for C_{1a} are derived from C_2 value measurements).

The modified NMR method described in this paper can provide a fast and acceptable estimation for the percent composition of gentamicins in commercial preparations. It is considerably shorter than the presently used official method (6), which relies on time-consuming paper chromatographic separation of the individual components followed by microbiological quantitation of the eluted fractions. Furthermore, certain commercial samples could not be assayed by the official method with confidence because of the presence of interfering materials in the samples.

References

(1) Weinstein, M. J., Luedemann, G. M., Oden, E. M., & Wagman, G. H. (1963) *Antimicrob. Agents Chemother.* 1
(2) Wilson, W. L., Richard, G., & Hughes, D. W. (1973) *J. Chromatogr.* **78,** 442–444
(3) Wilson, W. L., Richard, G., & Hughes, D. W. (1973) *J. Pharm. Sci.* **62,** 282–284
(4) Thomas, A. H., & Tappin, S. D. (1974) *J. Chromatogr.* **97,** 280–283
(5) Wagman, G. H., Oden, E. M., & Weinstein, M. J. (1968) *Appl. Microbiol.* **16,** 624–627
(6) Code of Federal Regulations, Title 21 Food and Drug 1980, 444.20
(7) Anhalt, J., Sancilio, F. D., & McCorkle, T. (1978) *J. Chromatogr.* **153,** 489–493
(8) Calam, D. H., Gilbert, J. N. T., Lightbown, J. W., Powell, J. W., & Thomas, A. H. (1978) *J. Pharm. Pharmacol.* **30,** 220–223
(9) Wagman, G. H., Marquez, J. A., Bailey, J. V., Cooper, D., Weinstein, J., Tkach, R., & Daniels, P. (1972) *J. Chromatogr.* **70,** 171–173
(10) *British Pharmacopoeia* (1980) Appendix IID, A59, 1405-41-0

FRUIT AND FRUIT PRODUCTS

Detection of Adulteration in Blackberry Juice Concentrates and Wines

RONALD E. WROLSTAD, JEFFREY D. CULBERTSON, CHRISTOPHER J. CORNWELL, and LEONARD R. MATTICK[1]
Oregon State University, Department of Food Science and Technology, Corvallis, OR 97331

Adulteration of blackberry juice concentrates and wines with juice of sorbitol-containing fruits was detected by determining carbohydrates by high performance liquid chromatography, gas-liquid chromatography, and thin layer chromatography. Sorbitol is not fermented by yeast and can be detected in wines made from blackberry juice concentrates that contain sorbitol. High levels of sorbitol and quinic and malic acids suggest that an imported blackberry concentrate may have been adulterated with plum. Degradation of anthocyanin pigments during processing and storage limits the utility of pigment analyses in detecting adulteration.

Berry juice concentrates have a higher economic value than apple, pear, or grape concentrates because of the higher cost of raw berries and lower processing yields. In 1978, the U.S. wholesale price for blackberry juice concentrate was $75 per gallon, whereas apple and pear juice concentrates sold for $6.25 per gallon. Effective screening methods are needed by processors and regulatory officials for detecting adulteration of juice concentrates with sugar, corn syrup, or less expensive fruit juice concentrates. Measurement of the $^{13}C/^{12}C$ ratio has been used to detect addition of corn syrup or cane sugar to honey (1) and apple juice (2), and presumably this method could be applied to other fruits such as blackberries which use the Calvin cycle for CO_2 fixation. Other methods are needed for confirmation and for detecting adulteration with beet sugar or other fruit juice concentrates.

Qualitative analysis of the sugars by gas-liquid chromatography (GLC) has been used (3) to detect adulteration of blackberry and other fruit juice concentrates. We have reported (4) the sugar and nonvolatile acid composition of 14 authentic blackberry samples by GLC determination of their trimethylsilyl (TMS) derivatives. Although there was considerable quantitative variation for the acids between samples, the sugar composition was quite uniform, with only glucose and fructose detected. Other workers in Europe and the United States (3, 5, 6) have reported that blackberries contain only glucose and fructose and do not contain sorbitol or sucrose. Sorbitol is present in cheaper concentrates such as apple, pear, and plum (7), which are readily available, and its presence could be an indication of adulteration with those fruits. A major use of blackberry concentrate is in wine production, and because sorbitol is not metabolized by yeast, it should be present after fermentation.

This study reports the analysis of imported and domestic blackberry juice concentrates and wines for adulteration by determining their sugar composition. High performance liquid chromatographic (HPLC) analysis offers a distinct advantage as a potential screening procedure because less time is required for sample preparation; this technique was applied in the analysis of several of the samples. GLC and thin layer chromatographic methods were also used for carbohydrate analyses. To confirm adulteration and attempt to identify the specific adulterant, analyses of nonvolatile acids by GLC and anthocyanin pigments by TLC were carried out for some samples.

METHODS

Samples

Imported blackberry juice concentrate (country of origin, Austria) purchased by a local processor was sampled at the plant when received. Domestic blackberry and plum concentrates were obtained from local processors. Commercial blackberry wines were purchased in a local market; labels declared that 2 of the wines were made from Oregon blackberries and one from imported European blackberries.

Two wines were prepared from concentrates B and C as follows: 143 g domestic concentrate B (71° Brix) and 154 g concentrate C (67° Brix)

[1] Department of Food Science and Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456.

Technical Paper No. 6246 from the Oregon Agricultural Experiment Station.
Received February 17, 1982. Accepted April 14, 1982.
This work was supported in part from a grant No. 10790054 from the Pacific Northwest Regional Commission.

0004-5756/82/6506-1417-07$01.00

were each combined with 400 g sucrose and diluted with water to 2 L. Inoculums were prepared from 0.5 g yeast (Fermivin, Code 5000C; GB Fermentation Industries, Inc.) and 50 mL of the appropriate blackberry juice concentrates diluted to 5° Brix, and were incubated 2 h at 34°C. Samples were inoculated and allowed to ferment for 47 days at 21°C. Samples were preserved with 20 ppm sodium metabisulfite.

Apparatus

(a) *High performance liquid chromatograph.*—Model 7000 (Micrometrics Instrument Co., Norcross, GA) fitted with 7.8 mm id × 30 cm Aminex HPX-87 cation exchange column, Ca form, 9 μm particle size (Bio-Rad Laboratories, Richmond, CA); Model R-401 differential refractive index detector (Waters Associates, Milford, MA); Recordall 5000 1 mV strip chart recorder (Fisher Scientific Co., Pittsburgh, PA); Model 18652A integrator (Hewlett-Packard Corp., Avondale, PA) combined with Computrend Model 43 computer. Operating conditions: mobile phase, deionized water; pressure, 590 psi; flow rate. 0.56 mL/min; column temperature 86.9°C; detector temperature, 20°C; chart speed, 0.5 cm/min; attenuation, × 64.

(b) *Gas chromatograph.*—Varian Aerograph Model 200 dual column gas chromatograph with hydrogen flame ionization detectors fitted with two 3 m × 2 mm id glass columns, one packed with 5% SE-53 on 80–100 mesh Chromosorb W and the other with 3% SE-30 on 80–100 mesh Chromosorb W; Hewlett-Packard Model 3380 A recording integrator. Operating conditions: Operate SE-52 column isothermally at 165°C for 14 min, then program at 12°/min to 250°C and hold. Program SE-30 column at 6°/min from 100 to 250°C and hold. Nitrogen carrier gas flow rate, 25 mL/min; injector temperature, 190°C; detector temperature, 250°C.

(c) *UV-visible spectrophotometer.*—Beckman Model DB-G recording double beam spectrophotometer.

Reagents

(a) *HPLC standard sugar solution.*—0.5% of each sugar. Add 0.50 g of each sugar (glucose, fructose, sucrose, and sorbitol) to 100 mL volumetric flask and dilute to volume with water.

(b) *pH 4.5 buffer.*—Combine 400 mL 1M sodium acetate and 240 mL 1N HCl. Adjust pH to 4.5 with same solutions and dilute to 1 L with water.

(c) *pH 1.0 buffer.*—Combine 125 mL 0.2N KCl and 385 mL 0.2N HCl, adjust to pH 1.0, and dilute to 1 L with water.

(d) *20% Potassium metabisulfite solution.*—Add 2.0 g $K_2S_2O_5$ to 10 mL volumetric flask; dissolve and dilute to 10 mL with water. Prepare fresh daily.

(e) *TLC solvent systems.* AHW, acetic acid–concentrated HCl–water (25 + 3 + 72); PAW, *n*-pentanol–acetic acid–water (2 + 1 + 1); ACMH, acetone–chloroform–methanol–water (80 + 10 + 10 + 15).

(f) *TLC visualization spray reagents—Molybdate reagent:* Adjust saturated aqueous solution of ammonium molybdate to pH 2 with 2N HCl, and filter. *Sugar reagent:* Spray TLC plate with DAP reagent (2 g diphenylamine, 2 mL aniline, 100 mL acetone, 10 mL phosphoric acid), and heat 30 min in 100°C forced air drying oven. *Polyol reagent:* Spray TLC plate with 0.1% sodium metaperiodate solution, and air-dry. Spray lightly with freshly prepared benzidine reagent (dissolve 1 g benzidine in 30 mL ethanol and dilute with 25 mL water, 11 mL acetone, and 0.5 mL 1N HCl).

Preparation of Samples for HPLC

Dilute fruit concentrate samples to 10° Brix with distilled water. Remove ethanol from 100 mL wine samples by boiling 5 min; cool and dilute to 100 mL with water. Degas wine samples not treated for ethanol removal in ultrasonic bath for 1 min. Apply 5 mL juice or wine sample to 6 mm id × 12 cm column containing 2 g AG1-X2, 200–400 mesh, anion exchange resin, OH-form (Bio-Rad). Wash with deionized water under 2–3 psi nitrogen pressure, and collect 50 mL eluant in volumetric flask. Regenerate column for subsequent samples by washing with following series of solutions: 25 mL 15% H_2SO_4, 25–50 mL deionized water, 5 mL 3N NaOH, 25 mL deionized water.

HPLC Determination of Sugars

Determine detector response factors (K) for individual sugars by external method. Inject 5 and 20 μL aliquots of HPLC standard sugar solution. Compute K factors from following formula:

$$K = (PA_2 - PA_1)/(W_2 - W_1)$$

where PA_2 and PA_1, and W_1 and W_2 are peak areas and weights (μg) of individual standard sugars injected.

Recompute K factors daily from 10 μL injection of HPLC standard sugar solution. (Typical K factors are sucrose, 2.96; glucose, 2.81; fructose,

Table 1. Sugar composition [a] of blackberry juice concentrates and wines, and plum juice concentrates

Sample	Fructose	Glucose	Sorbitol	Sucrose	Method
Blackberry concentrate A (domestic)	5.34	2.89	0	0	GLC
Blackberry concentrate B (domestic)	3.18	3.02	0.38	0.01	HPLC
Blackberry concentrate C (imported)	2.73	2.51	0.33	0.42	HPLC
Wine from blackberry concentrate B	0	0	0.24	0	HPLC
Wine from blackberry concentrate C	0.19	0.23	0.20	1.21	HPLC
Blackberry wine 1 (commercial)	2.86	3.54	0.165	7.52	HPLC
Blackberry wine 2 (commercial)	2.23	2.93	0	0.05	HPLC
Blackberry wine 3 (commercial)	2.98	3.61	0	0.04	HPLC
Plum concentrate A (domestic)	3.49	3.81	2.87	0	GLC
Plum concentrate B (domestic)	4.27	4.10	1.95	0	GLC

[a] g/100 mL; concentrates diluted to 10° Brix.

2.91; sorbitol, 3.02). Inject 20 μL sample. Compute individual sugars (μg) in sample injected by multiplying peak area by appropriate K factor. Sugar concentration, g/100 mL = μg sugar in sample × DF/μL sample injected × 10 where DF = dilution factor.

GLC Determination of Sugars and Acids

Dilute concentrate to 10° Brix; combine 50 mL diluted concentrate with 150 mL 95% ethanol, mix, and centrifuge at 2000 rpm. Decant supernatant and re-extract residue twice with 25 mL 80% ethanol. Combine washings and apply to cation exchange column (5 mL Bio-Rad AG 50W-X4, 200–400 mesh, H^+ form) connected in series to an anion exchange column (7 mL Bio-Rad AG 1-X8, 200–400 mesh, acetate form). Wash columns with deionized water until 2 L is collected in volumetric flask. Place 100 mL aliquot of this sugar fraction in a 250 mL volumetric flask and dilute to volume with water. Pipet 1 mL of this dilution into a 3 mL vial containing 100 μL 0.2% (w/v) rhamnose in 85% ethanol as an internal standard. Evaporate to dryness on a rotary evaporator (water bath, 35°C; vacuum, 736 mm Hg) and store under vacuum over P_2O_5 for at least 24 h.

Recover acids from anionic column by washing with 250 mL 10N formic acid followed by deionized water until 1 L eluant is collected in a volumetric flask. Evaporate 100 mL aliquot to dryness on rotary evaporator (water bath, 35°C; vacuum, 736 mm Hg), and dissolve residue in 10 mL distilled water. Pipet 1 mL into 3 mL vial containing 100 μL 1% (w/v) tartaric acid in 85% ethanol as an internal standard. Evaporate to dryness on rotary evaporator and store under vacuum over P_2O_5 at least 24 h.

Derivatize sugars by adding 300 μL Tri-Sil (Pierce Chemical Co., Rockford, IL), shake vigorously 5 min in Buchler shaker, heat 20 min at 70°C, shake an additional 15 min, and centrifuge. Derivatize acids by same procedure except heat 30 min at 50°C. Inject 2 μL sugar derivatives onto SE-52 column and 2 μL acid derivatives onto SE-30 column.

Calculate quantities of individual sugars by following formula:

$$\text{g/100 mL sample} = A_s/A_{is} \times W_{is}/K \times DF/R \times 2$$

where A_s = peak area of individual sugar; W_{is} = weight in mg of internal standard; DF = dilution factor. K (detector response factor) and R (% recovery) for individual sugars and acids are reported in a previous publication (8). Calculate quantities of individual acids by following analogous formula:

$$\text{meq acid/100 mL sample} = A_a/A_{is} \times W_{is}/K \times DF/R \times 2/\text{eq. wt}$$

where A_a = peak area of individual acid, and eq. wt is acid equivalent weight.

Determination of Anthocyanin Pigment Content

Dilute fruit juice concentrates to 10° Brix with water. Dilute wine and fruit juice samples with pH 1.0 buffer so that absorbance at 510 nm is less than 1.0. Dilute second sample to same strength with pH 4.5 buffer. Determine absorbance at wavelength of maximum absorbance (510 nm) and at 700 nm. Subtract absorbance at 700 nm (haze) from that at 510 nm. Determine difference in absorbance (A) at 510 nm (corrected for haze) between pH 1.0 and 4.5 samples. Calculate anthocyanin pigment concentrations (as cyanidin-3-glucoside) by following formula:

$$\text{Anthocyanin pigment (mg/L)} = A \times MW \times DF \times 10^3/\epsilon \times L$$

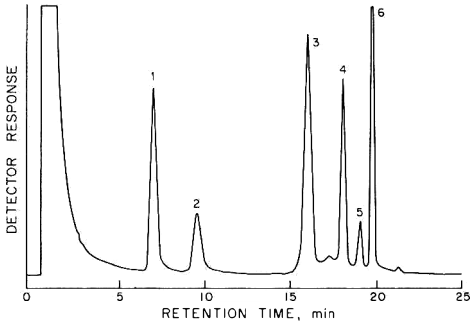


Figure 1. GLC separation on SE-52 column of TMS derivatives of sugars in imported blackberry juice concentrate: peaks 1 and 2, rhamnose (internal standard); peak 3, fructose; peak 4, α-glucose; peak 5, sorbitol; peak 6, β-glucose.

For cyanidin-3-glucoside, ϵ = 29 600 (9), and MW = 445; L = pathlength = 1.0.

Determination of Color Density, Polymeric Color, Percent Polymeric Color, and Browning Index

These methods are derived from procedures recommended by Somers (10). Dilute fruit juice concentrates to 10° Brix with water. Dilute wine and fruit juice samples with water so that absorbance at 510 nm (λ_{max}) is less than 1.0. Add 200 μL 20% potassium metabisulfite solution to 3.0 mL juice or wine sample. Add 200 μL water to a second 3.0 mL control sample. Determine absorbance at 420, 510 (λ_{max}), and 700 nm for both samples. Correct for haze by subtracting absorbance at 700 nm from values at 420 and 510 nm.

Color density

$$= A_{420} + A_{510} \text{ of control sample} \times DF$$

Polymeric color

$$= A_{420} + A_{510} \text{ of bisulfite-treated sample} \times DF$$

% Polymeric color

$$= (\text{polymeric color}/\text{color density}) \times 100$$

Browning index

$$= A_{420} \text{ of bisulfite-treated sample} \times DF$$

Isolation and TLC of Anthocyanin Pigments

Hydrate ca 35 g Polyclar AT (GAF Corp., New York, NY) overnight in water. Add to 3.0 cm id Buchner funnel lined with Whatman No. 1 filter paper. Apply volume of wine or juice sample that contains 15–25 mg anthocyanin pigment, taking care to avoid cracking Polyclar AT filter pad. Wash with water (volume = 10 × sample volume). Recover dark-colored anthocyanin-Polyclar AT adsorbate and place in column. Elute pigments with 0.1% methanolic HCl, washing until eluant shows faint color. Concentrate anthocyanin extract to dryness on rotary evaporator (water bath temperature, 35°C; vacuum, 736 nm Hg). Dissolve anthocyanin pigment in minimal volume of 0.1% methanolic HCl, and apply 1 μL to 20 × 20 cm × 0.25 mm cellulose MN 300 TLC plate (Anal Tech, Inc., Newark, DE). Develop first dimension in AHW (3 h), let plate air-dry to remove solvent, and develop second dimension in PAW (6 h).

TLC Detection of Sugars

Apply 1 μL wine or juice sample from HPLC analysis to 20 × 20 cm × 0.25 mm silica gel TLC plate, and also apply 2.5 μg of sugar standards (1 μL 0.25% standard sugar solutions). Develop in ACMH (2 h), air-dry, and repeat development 2 additional times. Detect sugars with sugar visualization spray and polyols with periodate-benzidine spray.

Results and Discussion

Table 1 shows the free sugar and sorbitol determined for 3 commercial blackberry juice concentrates. Sample A does not contain sorbitol, which is typical of authentic samples we have previously analyzed (4) and of other reports in the literature (5–7). Blackberry samples B and C contain substantial amounts of sorbitol, which is a clear indication that they are adulterated with some other fruit. Figure 1 is a GLC chromatogram of sugars in the imported blackberry juice concentrate. Sorbitol (peak 5) represents 5.5% of the total sugars.

Wines were produced from the 2 sorbitol-containing concentrates; their sugar content is also shown in Table 1. The wine produced from Sample B was fermented to dryness. The presence of sorbitol confirms that sorbitol is not fermented and can therefore be used as an index of adulteration in blackberry wines. The high residual sugar content of the wine produced from concentrate C indicates incomplete fermentation, either because of the presence of an inhibitor or the lack of an essential nutrient. The presence of sorbitol in the concentrate samples was confirmed by GLC and in the wine samples by TLC. Figure 2 shows the HPLC chromatograms for the concentrates and the wines produced after fermentation. A peak with the same retention as glycerol in the imported concentrate sample suggests that the raw material may have contained some partially fermented material before

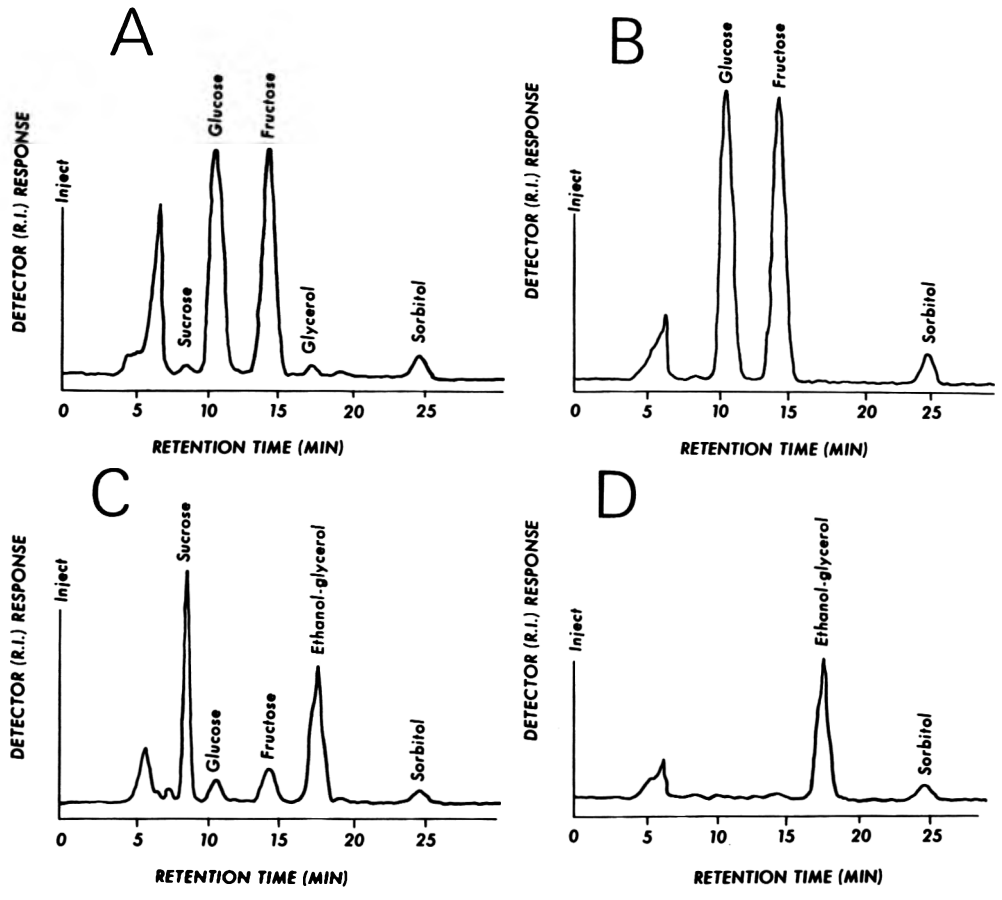


Figure 2. HPLC separations of sugars of blackberry juice concentrates and their derived wines on Aminex HPX-87 cation exchange column: A, blackberry juice concentrate C (imported); B, blackberry juice concentrate sample B (domestic); C, wine from blackberry juice concentrate sample C; D, wine from blackberry juice concentrate sample B.

concentration. The sugar composition of 3 commercial blackberry wines is also shown in Table 1. Commercial wine No. 1 (purported to be made from imported European blackberries) contains sorbitol; therefore, the authenticity of its fruit source is suspect.

The nonvolatile acid composition (Table 2) of domestic blackberry concentrate A is typical qualitatively and quantitatively of authentic blackberry samples we have previously analyzed (4). The imported blackberry concentrate, however, shows much higher levels of quinic acid. This suggests the possibility of plum juice being the adulterant because plums contain relatively large amounts of quinic acid as well as malic acid and sorbitol (Tables 1 and 2).

Blackberries are rich in anthocyanin pigment and have a relatively simple pigment pattern; cyanidin-3-glucoside and cyanidin-3-rutinoside are the major pigments that have been identified

Table 2. Nonvolatile acids[a] of blackberry and plum juice concentrates

Sample	Phosphoric	Malic	Lactoisocitric	Citric + isocitric	Quinic
Blackberry concentrate A (domestic)	1.32	1.45	0.71	6.08	0.06
Blackberry concentrate C (imported)	1.37	4.83	0	8.73	0.47
Plum concentrate A (domestic)	2.07	4.27	0	0.125	0.84
Plum concentrate B (domestic)	1.70	4.29	0	0.136	0.81

[a] meq/100 mL of 10° Brix concentrate.

Table 3. Anthocyanin pigment content and color properties of blackberry juice concentrates [a] and wines

Sample	Anthocyanin content, mg/L	Color density	Polymeric color	% Polymeric color	Browning Index
Blackberry concentrate B (domestic)	426	15.5	4.71	30.4	3.0
Blackberry concentrate C (imported)	30.1	16.3	11.8	72.3	7.0
Wine from concentrate B	48.1	4.35	2.1	48.3	1.3
Wine from concentrate C	18.0	7.9	6.0	75.9	3.5
Commercial wine 1	19.2	3.35	1.40	41.8	0.85
Commercial wine 2	50.4	2.80	1.50	53.6	1.0
Commercial wine 3	36.8	4.10	2.55	62.2	0.85

[a] Concentrate samples diluted to 10° Brix.

(11, 12). Plums contain the same 2 pigments, but also contain peonidin-3-glucoside and peonidin-3-rutinoside (12). The peonidin pigments do not change color with molybdate spray, which offers a simple method for checking their presence on a chromatogram. Figure 3 shows the TLC chromatograms of the anthocyanin pigments isolated from blackberry juice concentrates B and C and their respective wines. Only 2 pigments, tentatively identified as cyanidin-3-glucoside and cyanidin-3-rutinoside by co-chromatography with authentic standards and reaction with molybdate spray, were found in the imported blackberry concentrate sample. Although no peonidin pigments were found, the possibility of adulteration of that sample with plum still cannot be eliminated. The relatively low concentration of monomeric anthocyanin pigment and high amount of polymeric color and browning (Table 3) indicate that a large proportion of the anthocyanins in that sample had been degraded. Plums are not rich in anthocyanin pigment (1.9–53 mg/100 g fresh weight; 13) and the peonidin pigments could have been degraded and/or below the detection limits. Six anthocyanin pigments, all of which changed color with molybdate spray, were detected in concentrate B. Torre and Barritt (14) studied the anthocyanin composition of 19 blackberry varieties and reported the presence of unidentified pigments, many in trace quantities, in 11 of the samples. There are no conclusive differences between the chromatograms of pigment isolates of juice concentrates and their respective wines (Figure 3). The wine pigment isolates show more streaking and poorer resolution than do the juice concentrate isolates because of heavier sample loading and/or polymerization and degradation of pigments during processing and storage. The wine made from concentrate B contained some minor pigments in addition to the six which co-chromatograph with the pigment isolate from concentrate B. These may be detected because of greater sample loading, formation during fermentation and storage, or they may be artifacts of isolation or chromatography. Because of all these possibilities, caution must be used in attributing presence of trace quantities of pigments to adulteration.

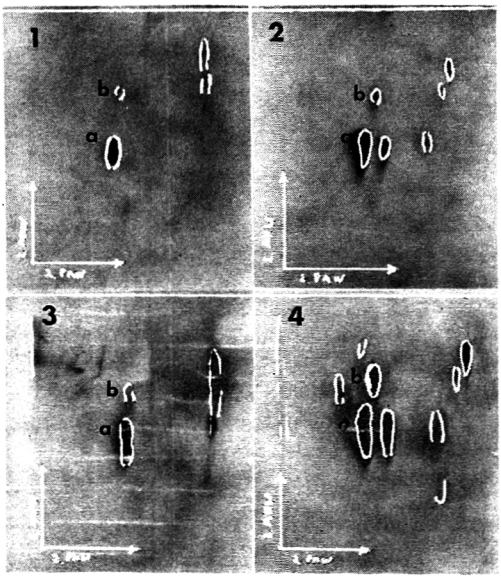


Figure 3. Photographs of 2-dimensional thin layer chromatograms of anthocyanin pigments from blackberry juice concentrates and their derived wines: 1, blackberry juice concentrate C (imported); 2, blackberry juice concentrate B (domestic); 3, wine from concentrate C; 4, wine from concentrate B. Spots a and b are tentatively identified as cyanidin-3-glucoside and cyanidin-3-rutinoside, respectively.

REFERENCES

(1) White, J. W., Jr, & Doner, L. W. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 746–750

(2) Doner, L. W., Krueger, H. W., & Reesman, R. H.

(1980) *J. Agric. Food Chem.* **28**, 362–364
(3) Fitelson, J. (1970) *J. Assoc. Off. Anal. Chem.* **53**, 1193–1197
(4) Wrolstad, R. E., Culbertson, J. D., Nagaki, D. A., & Madero, C. F. (1980) *J. Agric. Food Chem.* **28**, 553–558
(5) Benk, V. E., & Bergmann, R. (1978) *Flussige Obst* **45**, 14–17
(6) Kline, D. A., Fernandez-Flores, E., & Johnson, A. R. (1970) *J. Assoc. Off. Anal. Chem.* **53**, 1198–1202
(7) Wrolstad, R. E., & Shallenberger, R. S. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 91–103
(8) Akhavan, I., & Wrolstad, R. E. (1980) *J. Chromatogr.* **190**, 452–456
(9) Blundstone, H. A. W., & Crean, D. E. C. (1966) *The Pigments of Red Fruits*, The Fruit and Vegetable Preservation Research Association, Chipping Campden, UK
(10) Somers, T. C. (1972) *Food Technol. Aust.* **24**, 10–12
(11) Harborne, J. B. (1958) *J. Chromatogr.* **1**, 453
(12) Barritt, B. H., & Torre, L. C. (1973) *J. Chromatogr.* **75**, 151–155
(13) Van Buren, J. (1970) *The Biochemistry of Fruits and Their Products*, Vol. 1, Academic Press, New York, NY, Chapter 11, pp. 278, 280
(14) Torre, L. C., & Barritt, B. H. (1977) *J. Food Sci.* **42**, 488–490

RADIOACTIVITY

Determination of Radium-228 in Foods and Water

EDMOND J. BARATTA and ELLA M. LUMSDEN
Food and Drug Administration, Winchester Engineering and Analytical Center, Winchester, MA 01890

A procedure was developed for the rapid determination of ^{228}Ra by measuring its decay product ^{228}Ac. The method is applicable to a variety of types of samples such as food and water. Food samples can be prepared by dry- or wet-ashing techniques. The ^{228}Ac is precipitated with lanthanum carrier, dissolved, purified by solvent extraction, and finally decontaminated on anion and cation exchange resins. The ^{228}Ac is counted in a thin-window, low-level gas flow beta counter. It may be necessary to count the sample several times to follow the 6 h half-life of ^{228}Ac so that its purity is ensured. The method is sensitive to 1 pCi/sample of food ash and to 1 pCi/L of water.

^{228}Ra, a beta emitter, is found in soils and ores containing naturally occurring thorium nuclides. The detection of ^{228}Ra is difficult because the beta radiations emitted are weak, having maximum energies of 0.04 and 0.014 MeV. Its detection is also complicated by the presence of other radium isotopes such as ^{226}Ra, ^{223}Ra, and ^{224}Ra; these isotopes emit energetic alpha particles, and their short-lived decay products also emit beta radiations.

Most published methods for determining ^{228}Ra are complex, may require specialized instrumentation and/or long ingrowth periods, and are suitable only for water (1–7). The National Bureau of Standards (8) conducted a survey of methods for determining ^{228}Ra and found that few of those surveyed gave procedures for assaying water; in addition, results of such measurements were not always given. Two methods (1, 4) were adequate, but either they have limitations or sophisticated counting equipment was needed. These two were, moreover, tested only for water. Two other methods (9, 10) listed were believed to have the sensitivity needed but had not been tested fully. The latter method (10) was originally applied to ^{227}Ac, and ^{228}Ac was considered of secondary importance.

Received February 12, 1982. Accepted April 21, 1982.
This paper was presented at the 94th Annual Meeting of the AOAC, Oct. 20–23, 1980, at Washington, DC.

A procedure, based on the previous method for ^{227}Ac (10), has been developed in this laboratory for the rapid determination of ^{228}Ra in foods and water. The method can be used to determine ^{228}Ra levels as required by the Code of Federal Regulations (11), the Environmental Protection Agency's (EPA) Drinking Water Standards (12), and the Food and Drug Administration's (FDA) regulations on bottled water (13). The method was tested with a fairly difficult food sample (black pepper) and at levels necessary to meet the EPA Drinking Water Standards.

^{228}Ra is determined by measuring its decay product, ^{228}Ac, that has reached a known secular equilibrium stage with a 6.1 h half-life. This procedure involves the isolation and measurement of the ^{228}Ac which has an energetic maximum beta emission of 2.1 MeV.

The method depends on the separation of ^{228}Ac from contaminating radium nuclides as well as from lead, polonium, and bismuth daughter products. The interference from ^{227}Ac, a daughter of ^{235}U, is insignificant because this nuclide has a weak maximum beta emission of only 0.047 MeV, making it very difficult to detect through the 500 μg/sq. cm window of the beta counter. The method consists of the rapid determination of the naturally occurring beta emitter ^{228}Ac and is based on a combination technique of solvent extraction and ion exchange. No stable actinium is found present in nature; therefore a co-precipitate is necessary. As shown in previously developed methods, the actinides follow the chemistry of the lanthanides; for this reason, lanthanum was used as the carrier or co-precipitant.

The procedure is designed for analysis of natural waters and various foods such as Total Diet composites, fruits, spices, tea, and other food items. It is of radiobiologic interest because ^{228}Ac is also a measure of ^{228}Ra which, if found in foodstuffs and ingested, is hazardous because of the high energetic beta emission of its immediate daughter product ^{228}Ac and the subsequent alpha-emitting decay products (13). The method is sensitive to less than 1 pCi/sample of food ash

0004-5756/82/6506-1424–05$01.00

and to the levels required by the EPA Drinking Water Standards (1 pCi/L). Four solubilized samples can easily be processed in 4–6 h.

Experimental

Principle

This solvent extraction-ion exchange procedure for the separation of actinium is suitable for various foods and natural waters. With modifications, it can be applied to other types of samples.

Food samples are normally ashed before solubilization, and the ^{228}Ac, because of its short half-life, is in secular equilibrium before the sample is analyzed. Collection times of water samples are usually known and the ^{228}Ac equilibrium value can be easily calculated. Water samples are preserved in the conventional manner (12).

Actinium is initially precipitated with lanthanum fluoride to separate it from the bulk of the cations including radium; however, thorium and uranium are not removed. To remove most of the uranium, the precipitate is treated with dichromate, which keeps uranium soluble in the hexavalent state. The solution is passed through an anion exchange column to complete the removal of lead, bismuth, polonium, and any remaining uranium. Thorium, which has been carried quantitatively to this point, is removed by extracting the solution with 2-thenoyltrifluoracetone (TTA)-benzene in the presence of aluminum nitrate. As a final decontamination step, the solution is passed through a cation exchange column and the actinium is selectively eluted by citric acid; any remaining radium or thorium is retained on the resin.

Reagents

(a) *2-Thenoyltrifluoracetone in benzene.*—0.5M. (Note: Benzene is hazardous and a known carcinogen; toluene may be substituted if necessary.)

(b) *Lanthanum nitrate.*—12 mg lanthanum/mL water.

(c) *Oxalic acid.*—7% in water.

(d) *Citric acid.*—5% in water, adjusted to pH 5.0.

(e) *1.0M Hydrofluoric acid + 1.0M nitric acid.*—1 + 1.

(f) *Aluminum nitrate.*—2.0M in water.

(g) *Potassium dichromate.*—Saturated.

Apparatus

(a) *Chromatographic columns.*—20 cm long × 1 cm id.

(b) *Filtration apparatus.*—Two-piece, stainless steel (or Teflon, Fluorolon Laboratory, Caldwell, NJ) with 1 in. nylon ring and disk mounts (Control Molding Corp., Staten Island, NY).

(c) *Mylar film.*—1 in. × 0.25 mil (E.I. DuPont de Nemours, Wilmington, DE).

(d) *Centrifuge tubes.*—Conical, plastic, 50 mL.

(e) *Filter paper.*—Glass fiber, diameter 2.4 cm (No. 934-AH, Whatman, Inc., Clifton, NJ).

(f) *Flow counter.*—Wide Beta II, with 1¼ in., 500 μg/sq. cm window (Beckman Instruments, Inc., Irvine, CA).

Column Preparation

Prepare anion exchange column by adding Dowex 1-X8 (100–200 mesh, chloride form, Dow Chemical Co., Indianapolis, IN) in 1.8M HCl to a height of ca 7 cm to 20 cm column. Wash column with several column volumes of 1.8M HCl.

Prepare cation exchange column by adding Dowex 50-X8 (100–200 mesh, hydrogen form, Dow Chemical Co.) in 0.2M HNO_3 to height of ca 7 cm to 20 cm column. Wash column with several column volumes of 0.2M HNO_3.

Columns are packed to allow liquids to flow through rapidly, because flow rate is not critical.

Sample Solubilization

Ash food sample of appropriate size by either wet- or dry-ashing. Dissolve ashed sample in 4M HCl with the aid of heat (use fume hood). Remove any dirt particles in dissolved sample by filtering through glass fiber paper in Buchner funnel. Collect filtrate and dilute to 500 mL with water and 4M HCl so that final concentration is 1M HCl.

If sample is known to contain large amounts of interfering ions such as iron, clean up as follows. Add 1 mL $BaCl_2$ carrier (20 mg/mL) to sample and then precipitate barium with 10 mL $(NH_4)_2SO_4$ (50% w/v). After $BaSO_4$ is well settled, discard supernate and transfer precipitate to test tube.

Add 20 mL Na_2CO_3 to precipitate and heat in boiling water bath, with occasional stirring to aid conversion of $BaSO_4$ to $BaCO_3$. Dissolve $BaCO_3$ in 1M HCl. Set aside for 36 h for ingrowth (98.3% equilibrium between ^{228}Ra and ^{228}Ac).

Procedure

To a solubilized sample in tube or at least 1 L water, add 1 mL lanthanum nitrate carrier. Add enough concentrated HF (ca 5 mL) to precipitate lanthanum as fluoride, and stir 10 min. Let

precipitate settle and decant most of the liquid. Centrifuge (200 rpm), and make all required washings (after each centrifugation) with 1M HF + 1M HNO_3. Make final collection in 50 mL plastic centrifuge tube.

Add several drops of saturated $K_2Cr_2O_7$ solution and 1 mL concentrated HF to precipitate. Dilute to ca 20 mL with water and digest 5 min. Centrifuge, discard supernate, and wash precipitate with 20–30 mL HF–HNO_3. Repeat washings until precipitate is white, discarding supernates. Add 20 mL 19M NaOH and digest 5 min. Centrifuge and discard supernate. (Wash with 19M NaOH, if necessary, to remove excess contaminating precipitate such as CaF.) Add several drops of 1M NaOH to actinium hydroxide precipitate and wash with ca 20 mL water. Centrifuge and discard supernate.

Dissolve precipitate in ca 10 mL 1.8M HCl and pass solution through previously prepared anion exchange column, collecting solution in 40 mL glass centrifuge tube. Wash column with 1 column volume of 1.8M HCl and combine with solution.

Add concentrated NH_4OH to reprecipitate actinium–lanthanum as hydroxides. Centrifuge, discard supernate, and wash precipitate with water containing several drops of 1M NH_4OH; centrifuge and again discard supernate.

To precipitate, add no more than 1 drop of concentrated HNO_3, 5–10 mL 0.2M HNO_3, and 1 mL 2.0M aluminum nitrate. Adjust to pH 1.0 with NaOH, if necessary. Transfer to 125 mL separatory funnel with 0.2M HNO_3 washings. Add 10 mL TTA–benzene (0.5M) and shake 5 min. Let layers separate and discard benzene layer. If sample contains large amounts of thorium, as indicated by a deep reddish color, repeat extraction step.

Add entire aqueous phase to previously prepared cation exchange column, making sure funnel stem has been washed with 0.2M HNO_3. Let solution pass through column and wash column with 1 column volume of 0.2M HNO_3. Discard solution and washing. Elute with 5% citric acid (pH 5.0), discarding first column volume and collecting next 30 mL.

To 30 mL eluate, add 10–12 mL 7% oxalic acid to reprecipitate lanthanum–actinium as oxalate and centrifuge. Wash precipitate with water containing several drops of 7% oxalic acid and centrifuge.

Weigh 2.4 cm glass fiber filter and place on filtration apparatus. Take up precipitate in 10 mL water and transfer to filter paper with water. Wash precipitate with three 10 mL portions of absolute ethanol. Continue suction until alcohol is removed from precipitate. Remove filter from apparatus and dry in 125°C oven 20 min. Cool to room temperature and weigh to determine chemical yield.

Place filter on Mylar disk, cover with Mylar, and seal with ring. Count beta activity of ^{228}Ac in low background beta counter. Record time counting is started for use in determining midpoint of ^{228}Ac decay so that decay factor from time of separation to midpoint can be calculated.

^{228}Ra activity is calculated according to the following equation.

^{228}Ra (pCi/sample) = (gross cpm − B)/(2.22 × $C \times D \times E \times W$) where B is detector background (including filter), C is chemical yield, D is detector geometry, E is decay factor for ^{228}Ac between separation and counting, and W is weight in kg of original food sample or liter of water.

Note: ^{228}Ac decay may be followed by counting several times and, if necessary, the following day.

Results and Discussion

The method was tested by adding various radionuclides such as natural uranium, ^{230}Th, ^{90}Y, ^{226}Ra, ^{210}Pb, ^{210}Bi, ^{210}Po, and ^{90}Sr to water samples. Table 1 shows the radionuclide added, the

Table 1. Removal of radionuclides from actinium

Radionuclide	Removal procedure	Added, dpm	Recovered, dpm	Decontamination factor
U (natural)	$K_2Cr_2O_7$	11 400	40 ± 0.6	285
^{230}Th	TTA-benzene	3 400	12 ± 0.4	285
^{90}Y	TTA-benzene	1 000	4 ± 0.5	278
^{226}Ra	Cation column	5 400	14 ± 0.4	385
^{210}Pb	Anion column, 1.8M HCl	22 600	26 ± 0.5	870
^{210}Bi	Anion column, 1.8M HCl	20 000	25 ± 0.5	770
^{210}Po	Anion column, 1.8M HCl	140 000	15 ± 0.4	9 300
^{90}Sr	Cation column	1 000	4 ± 0.5	278

Table 2. Recovery of ^{228}Ac from ashed pepper samples containing added ^{228}Ra and lanthanum carrier [a]

	Activity recovered		Carrier recovered	
Sample	mg	%	dpm	%
1	16.9	84.5	10.9	83.5
2	17.6	88.0	11.1	85.0
3	16.4	82.0	10.6	81.8
4	16.0	80.0	10.9	83.8
5	16.8	84.0	10.7	82.6
6	16.3	81.5	11.0	84.8
7	16.6	83.0	11.0	84.8
Mean ± 1 SD		83.3 ± 2.6		83.8 ± 1.2

[a] Amounts added to each sample were 13 dpm ^{228}Ra and 20 mg lanthanum carrier. Samples were counted for 100 min; background was 0.5 cpm.

method or the decontamination step of the procedure used, the amount recovered, and the decontamination factors obtained. The amount of activity recovered demonstrated that the decontamination was more than sufficient to prevent interference with contaminants at levels normally encountered.

To ensure that the method was applicable to foods and water, black pepper was chosen as the matrix to be tested. This product is known to contain natural uranium and ^{226}Ra. Previous experience has shown that pepper is a difficult food sample to analyze, because it also contains large amounts of salts including iron. Enough pepper was ashed to provide samples for 7 determinations. The ash of each sample was dissolved in 4M HCl and diluted so that the final concentration was 1M HCl. Lanthanum carrier and a ^{228}Ra standard (obtained from the Quality Assurance Division, EPA, Las Vegas, NV) were added, and the carrier and ^{228}Ac were determined. Table 2 shows the recovery, both in milligrams and percentage for the carrier, for the analyses of the 7 pepper samples. The average percent recovery for the carrier was 83.8 ± 1.2 for 1 standard deviation (SD). The recovery for the activity (Table 2) was 83.3 ± 2.6 (1 SD). The data show that the method is consistent with the recovery obtained by using the carrier (lanthanum) and the added ^{228}Ra tracer. Considering the matrix involved and the decontamination necessary, the method is adequate and consistent.

This method meets the necessary requirements to obtain levels of ^{228}Ra at 1 pCi/sample by measurement of its decay product ^{228}Ac. The low-background beta counter used in these measurements has a historic background (a glass fiber filter mounted on a Mylar ring and disk and covered with Mylar) of 0.52 ± 0.09 cpm for a 100 min count. The efficiency was determined to be about 45% for this type of detector with a 500 μg/sq. cm window. With a recovery of about 80% and even with a decay factor of 50% and counting time of 100 min, the counting error was calculated to be 0.3 pCi/sample. Because of this possible error, the sample size for water may have to be increased to 4 L, if necessary. The decontamination factors are high for interfering radionuclides and should not present any problems. The technique does not require specialized equipment, and the analysis is fairly rapid and can be applied to a variety of samples.

REFERENCES

(1) McCurdy, D. E., & Mellor, R. A. (1979) 24th Annual Meeting of the Health Physics Society, July 8–13, 1979, Philadelphia, PA

(2) Humphrey, H. W., Adkins, E. L., & Templin, G. G. (1975) *The Radiometric Determination of ^{226}Ra and ^{228}Ra in Waste Effluent Solutions*, Report NLCO-1084, U.S. Atomic Energy Commission, Washington, DC

(3) Johns, F. B., Hahn, P. B., Thome, D. J., & Brethauer, E. W. (1979) *Radiochemical Analytical Procedures for Analysis of Environmental Samples*, Report EMSL-LV-0539-17, U.S. Environmental Protection Agency, Las Vegas, NV

(4) Krieger, H. L. (1976) *Interim Radiochemical Methodology for Drinking Water*, Report EPA-600/4-75-008 (Revised), U.S. Environmental Protection Agency, Cincinnati, OH

(5) Johnson, J. O. (1977) *Determination of Radium-228 in Natural Waters*, Water-Supply Paper 1696-G, U.S. Geological Survey, Washington, DC

(6) Moore, W. S., & Reid, D. F. (1973) *J. Geophys. Res.* **78**, 8880–8886

(7) Michel, J., Moore, W. S. & King, P. T. (1981) *Anal. Chem.* **53**, 1885–1889

(8) *Evaluation of Methods for Assay of Radium-228 in Water* (1981) NBS Technical Note 1137, National Bureau of Standards, U.S. Department of Commerce, Washington, DC

(9) Iyer, R. H., Jain, H. C., Ramaniah, M. V., & Rao, C.

L. (1966) *Radiochim. Acta* 5, 56
(10) Baratta, E. J., & Feldman, M. H. (1961) *Determination of Actinium in Uranium Mill Effluents*, USAEC Topical Report WIN-123, U.S. Atomic Energy Commission, Washington, DC
(11) *Code of Federal Regulations* (1980) Title 10, U.S. Government Printing Office, Washington, DC, Part 20
(12) *National Interim Primary Drinking Water Regulations* (1976) EPA-570/9-76-003, U.S. Environmental Protection Agency, Washington, DC
(13) *Fed. Regist.* (1979) 44(45), 12169

MYCOTOXINS

Capillary Gas-Liquid Chromatographic Determination of Vomitoxin in Cereal Grains

HUGUETTE COHEN and MICHEL LAPOINTE
Agriculture Canada, Food Production and Inspection Branch, Ottawa, Ontario, Canada K1A 0C5

A gas-liquid chromatographic (GLC) method, using an electron capture detector, is described for determining vomitoxin in cereal grains at levels as low as 0.05 ppm. Samples are extracted with chloroform-ethanol and initially purified by using a Sep-Pak silica cartridge, followed by column chromatography using Sephadex LH-20. Additional purification is achieved by using a disposable cyano cartridge. Heptafluorobutyrylimidazole (HFBI) is added to form esters of the analytes. The HFB esters of vomitoxin are separated on a 30 m × 0.25 mm fused silica column coated with SE-54 and measured with a ^{63}Ni electron capture detector. Samples were confirmed by gas-liquid chromatography/mass spectrometry using electron impact ionization and single ion monitoring at the molecular ion mass of 884.05 and at 10 000 resolving power. The method has been applied to wheat, oats, barley, and corn. The limit of detection is 0.05 ppm vomitoxin. Average recoveries were 89.8% with a coefficient of variation of 4.0%.

Vomitoxin or deoxynivalenol belongs to the trichothecene family of mold metabolites produced by various strains of *Fusarium, Trichoderma, Mycothecium*, and other species of imperfect fungi. The literature contains information on 23 trichothecenes produced by *Fusarium* under laboratory conditions (1-4), but only 7 of these trichothecenes are produced in significant yields by various strains of *Fusarium*. Among these, deoxynivalenol (DON) or vomitoxin has been associated with feed refusal, vomiting, and general digestive disorders in animals ingesting moldy grain or feed.

Most determination procedures published in the literature involve a solvent extraction and a cleanup involving either liquid-liquid partition with purification by thin layer chromatography or silica gel column before derivatization, or gas-liquid chromatographic analysis (GLC) (5-8). For GLC using electron capture detection with the reagent heptafluorobutyryl imidazole to form the DON tris-heptafluorobutyrate (9), sensitivity is greater. The detection limit is lower with an electron capture detector for the halogenated derivative than with the flame ionization detector for the silyl ether derivatives.

The proposed method involves extraction with chloroform–ethanol (80 + 20) followed by purification by using a Sep-Pak silica cartridge and a Sephadex LH-20 column with the incorporation of a cyano cartridge for levels lower than 0.20 ppm before derivatization.

METHOD

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard Model 5880 equipped with electron capture detector and 30 m × 0.25 mm id fused silica capillary column coated with SE-54, 0.25 μm film thickness. Detector attenuation set at 2×10^{-7} AFS and 1 mV strip chart recorder.

(b) *Tube mixer.*—Thermolyne Maxi-Mix.

(c) *Glass chromatographic columns.*—25 cm × 0.7 cm id with 50 mL reservoir.

Reagents

(a) *Solvents.*—All distilled in glass (Caledon, Georgetown, Ontario).

(b) *Adsorbents.*—Sephadex LH-20 (Pharmacia, Montreal); Sep-Pak silica cartridge (Waters Associates); Cyano extraction column (J. T. Baker).

(c) *Vomitoxin.*—Available from Myco Lab Co., PO Box 321, Chesterfield, MO 63017.

(d) *Heptafluorobutyrylimidazole (HFBI).*—Pierce Chemical Co., Rockford, IL.

Sample Preparation

Grind 2-3 lb representative sample to pass 2 mm screen. Mix thoroughly until 100 g representative sample is prepared. Further mix 100 g; then weigh 10 g into 500 mL Erlenmeyer flask.

0004-5756/82/6506-1429-06$01.00

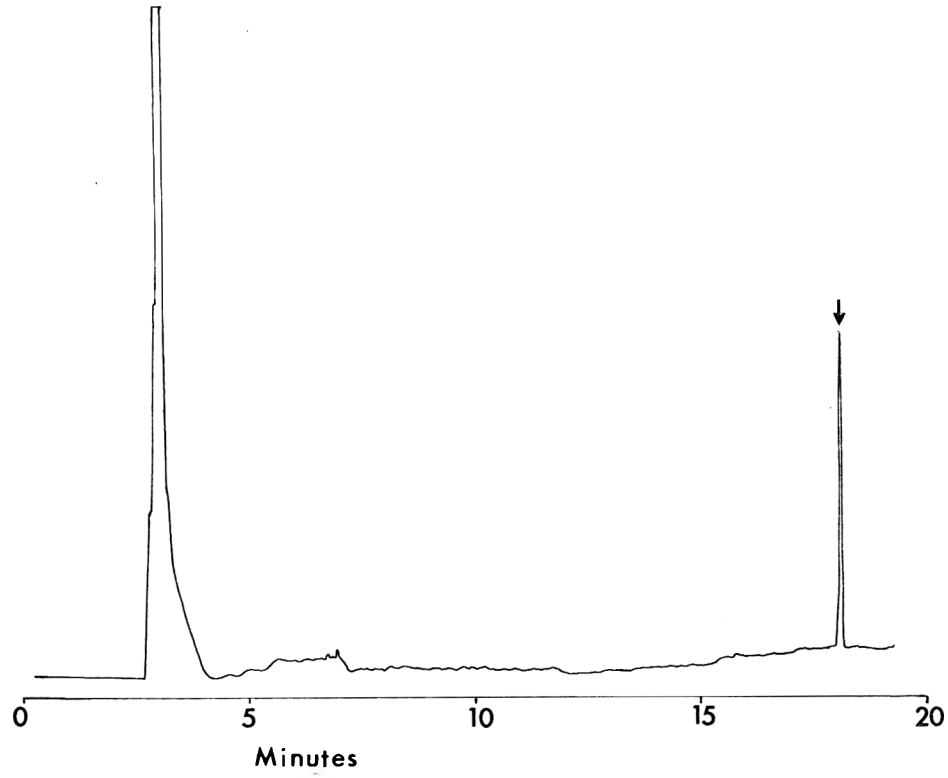


Figure 1. Chromatogram of 45 pg vomitoxin tris-heptafluorobutyrate (arrow).

Extraction and Cleanup

Shake 10 g sample 1 h with 200 mL chloroform–ethanol (8 + 2) on wrist-action shaker. Filter mixture into 500 mL round-bottom flask. Wash filtrate with an additional 50 mL chloroform–ethanol (8 + 2). Evaporate organic solvent to dryness on rotary evaporator. Dissolve residue in 2 mL dichloromethane and transfer to 50 mL glass syringe to which is attached silica gel Sep-Pak cartridge. Wash round-bottom flask with three 5 mL portions of dichloromethane and quantitatively transfer rinses to cartridge.

Table 1. Recovery of vomitoxin from wheat and barley [a]

Sample	Amt added, ppm	Found, %	SD	CV, %
Wheat	3.6	87.1	0.9	1.0
Barley	1.8	87.9	5.6	6.4
Wheat	0.9	96.6	2.9	3.0
Wheat	0.56	88.5	4.9	5.5
Wheat	0.11	87.7	9.7	10.9
Wheat	0.056	91.5	9.2	10.0

[a] All results are average of 3 samples spiked at that level.

Discard first 10 mL dichloromethane. Add 25 mL methanol–dichloromethane (5 + 95) and elute vomitoxin from cartridge into small round-bottom flask. Evaporate organic solvent from vomitoxin fraction, using rotary evaporator. Prepare 20 cm × 0.7 cm id column of Sephadex LH-20 by slurrying LH-20 in dichloromethane–methanol (8 + 2) and pouring into glass column to height of 18 cm. Quantitatively transfer vomitoxin fraction from cartridge to top of LH-20 column with 10 mL methanol–dichloromethane (5 + 95). Discard this fraction. Add 30 mL methanol–dichloromethane (2 + 8) to top of column, collect eluate, and evaporate to dryness. Dilute to 10 mL with toluene–acetonitrile (95 + 5). Transfer 1g sample to Cyano extraction column. Add an additional 9 mL toluene–acetonitrile (95 + 5) and discard this first fraction. Elute with 15 mL toluene–acetonitrile (80 + 20) into 15 mL graduated centrifuge tube and keep this fraction for vomitoxin analysis.

Derivatization

Evaporate to dryness and add 100 μL HFBI to sample; mix well using tube mixer. Dilute to 1

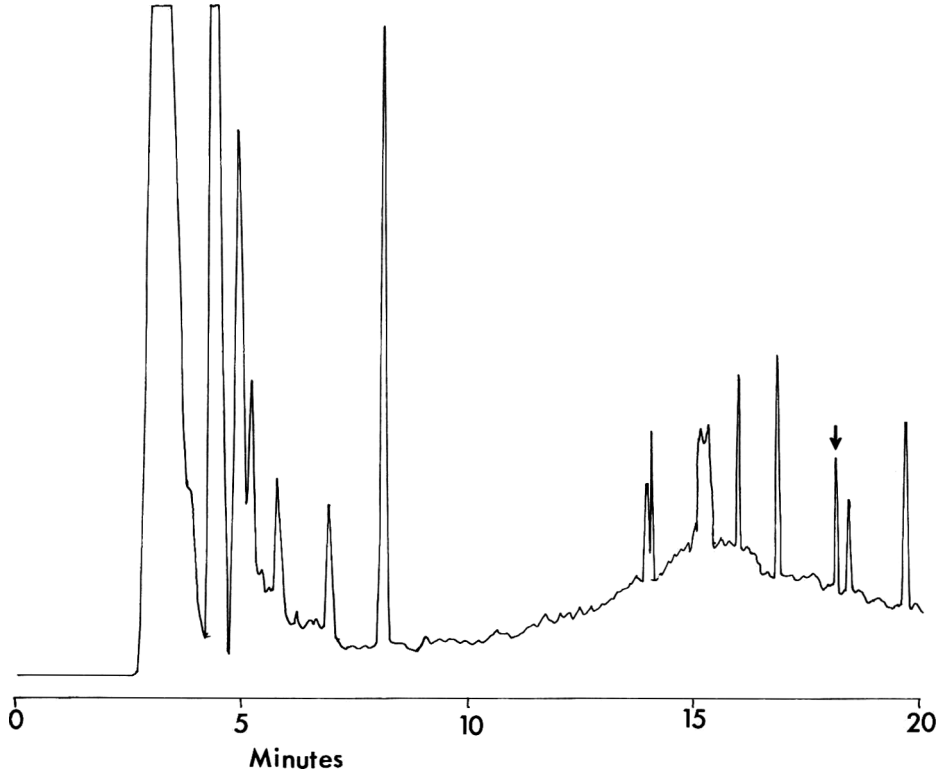


Figure 2. Chromatogram of extract of naturally contaminated Ontario winter wheat; 2 μL of 0.2 g equivalent wheat/mL corresponding to 0.04 ppm.

mL with toluene–acetonitrile (95 + 5). Heat tubes 1 h in sand bath at 60°C. Cool and mix again. Add 1 mL 5% aqueous sodium bicarbonate solution and mix 2 min. Let stand to separate layers. Transfer 100 μL organic layer, using 100 μL syringe, to 5 mL graduated centrifuge tube and dilute to 1 mL with heptane.

Table 2. Vomitoxin levels in 1980 winter wheat, barley, corn, and oats [a]

Sample	Province	Level, ppm
Wheat	Ontario, London	0.45
Wheat	Dresden	0.60
Wheat	Hyde	0.10
Barley	St. Thomas	0.24
Barley	Woodstock	0.26
Barley	Moffat	0.43
Corn	Quebec	0.47
Corn		0.79
Corn		0.89
Oats	Quebec, Thurso	0.11
Oats	Thurso	0.08
Oats	Papineauville	0.11

[a] Small cross-section of more than 300 samples analyzed.

Standard Solutions

(a) *Initial standard solutions.*—Prepare vomitoxin standard equivalent to 1 mg/10 mL ethyl acetate.

(b) *Working standard solution.*—Transfer 10 μg vomitoxin to 5 mL graduated centrifuge tube. Dilute to 2 mL with toluene–acetonitrile (95 + 5). Add 100 μL HFBI and carry out reaction as before. Transfer 50 μL derivatized vomitoxin to 10 mL volumetric flask. Dilute to volume with heptane to give vomitoxin standard of 25 pg/μL.

Gas Chromatographic Analysis

If possible, carry out analysis on same day as derivatization, using the following oven temperature profile:

Initial value	100°C
Initial time	2.00 min

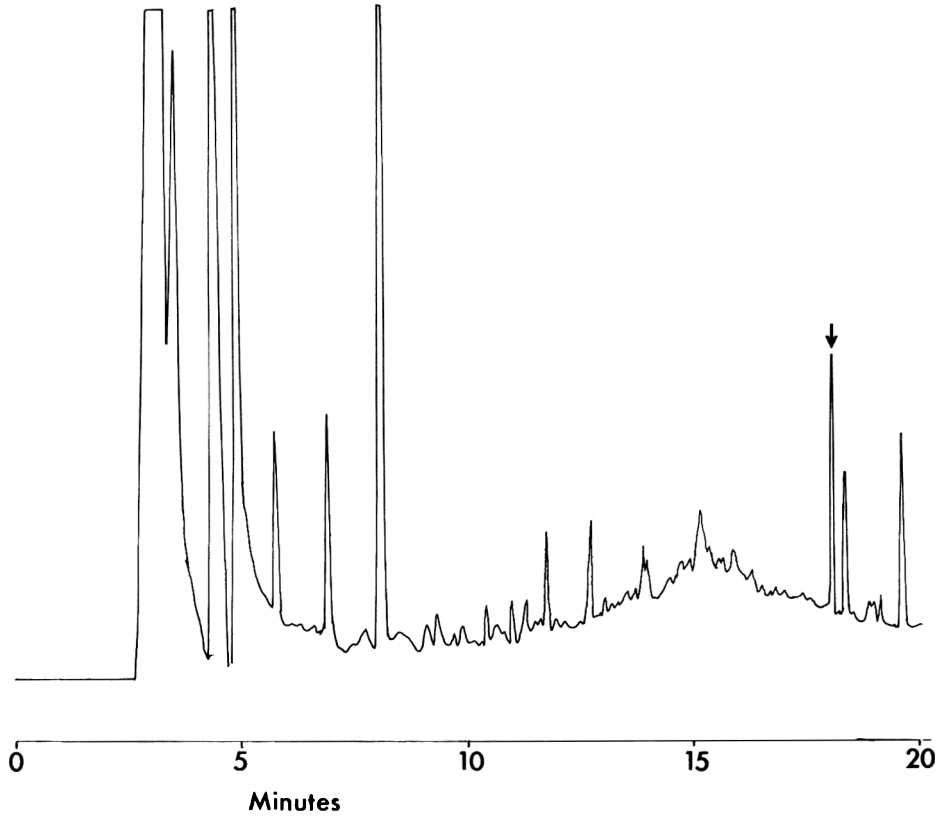


Figure 3. Chromatogram of extract of spiked Ontario winter wheat; 2 μL of 0.1 g equivalent wheat/mL corresponding to 0.11 ppm.

Level 1	
Program rate	10.00°C/min
Final value	250°C
Final time	5.00 min
Level 2	
Program rate	25.00°C/min
Final value	300°C
Final time	5.00 min

Additional conditions: Injector 200°C; detector 300°C; helium carrier gas linear velocity 35 cm/s; make-up gas (5% methane, 95% argon) flow rate 40 mL/min; chart speed 1 cm/min. Vomitoxin is chromatographed during level 1 of program. Level 2 of program ensures that late-eluting peaks will not interfere with analysis of subsequent samples. Keep injection volumes at ≤2 μL. Plot different amounts of HFB vomitoxin to obtain analytical calibration curve daily. Retention time of derivatized vomitoxin is 18.35 min. Quantitate by comparison with standard peak heights. Peak height is linear with concentration for the range 10 to 100 pg. Minimum detectable amount at attenuation 2×10^{-7} AFS is 10 pg with signal-to-noise ratio better than 10:1. All injections were done using the splitless mode with the valve open for 0.75 min before closing.

Confirmation

Vomitoxin was confirmed by GLC/MS with a Perkin Elmer Sigma III chromatograph interfaced to a Kratos MS-50 mass spectrometer with a single stage glass jet separator. A 6 ft × 1/4 in. glass column packed with 3% SE-30 Ultraphase on 80–100 mesh chrome 750 operated isothermally at 220°C with a helium flow of 30 mL/min was used for chromatographic separation. High level and low level quantitation in samples was carried out by single ion monitoring at the molecular ion mass of 884.05, and 10 000 resolving power.

Results and Discussion

Chloroform–methanol (80 + 20) was chosen

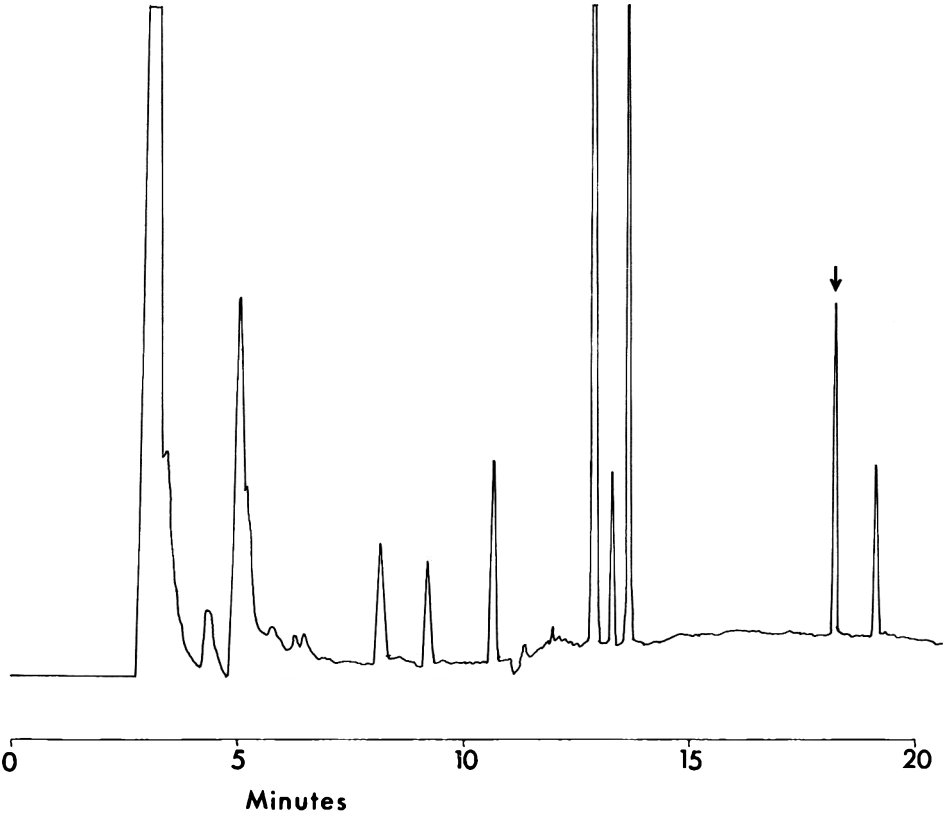


Figure 4. Chromatogram of extract of naturally contaminated Ontario winter wheat; 2 μL of 0.02 g equivalent wheat/mL corresponding to 1.4 ppm.

as extraction solvent because recoveries done using this solvent system were in excess of 85%. In contrast to aqueous extraction solvents (9–11) which gave a yield of 60%, the use of chloroform–ethanol also eliminated the use of a partition which often leads to emulsion problems.

The use of a Sep-Pak silica cartridge was a quick and easy initial purification before chromatography on Sephadex LH-20.

The vomitoxin fraction eluting from Sephadex LH-20 and reacted with HFBI was suitable at this point for GLC analysis of samples above 0.20 ppm. At levels below 0.2 ppm, an extra purification step was introduced, using the cyano cartridge to purify the sample after LH-20 treatment. With this extra step, levels of 0.05 ppm could be attained.

Derivatization was consistent in the toluene–acetonitrile mixture. Reaction was complete in 60 min at 60°C and sensitivity was such that 10 pg vomitoxin could easily be detected. Aqueous sodium bicarbonate was used to remove excess HFBI which in itself produces a response by the EC detector.

Figure 1 illustrates a typical chromatogram of 45 pg vomitoxin. Figure 2 is a chromatogram of an animal feed before spiking found to contain 0.04 ppm, and Figure 3 is a chromatogram of an animal feed sample spiked at 0.11 ppm. Figure 4 is a chromatogram of a naturally contaminated wheat sample in which 1.4 ppm of vomitoxin was detected.

Representative recoveries of vomitoxin added to cereals at levels of 3.6–0.056 ppm are shown in Table 1. All samples were analyzed in triplicate along with a separate blank. Overall recoveries were consistently in the range of 89.8% ± 3.6. Table 2 gives results from naturally contaminated field samples of a variety of cereal crops showing the presence of vomitoxin.

Figure 5 is the GLC/MS trace for single ion monitoring at the molecular ion mass of 884.05 at 10 000 resolution of a pure standard and a naturally contaminated sample.

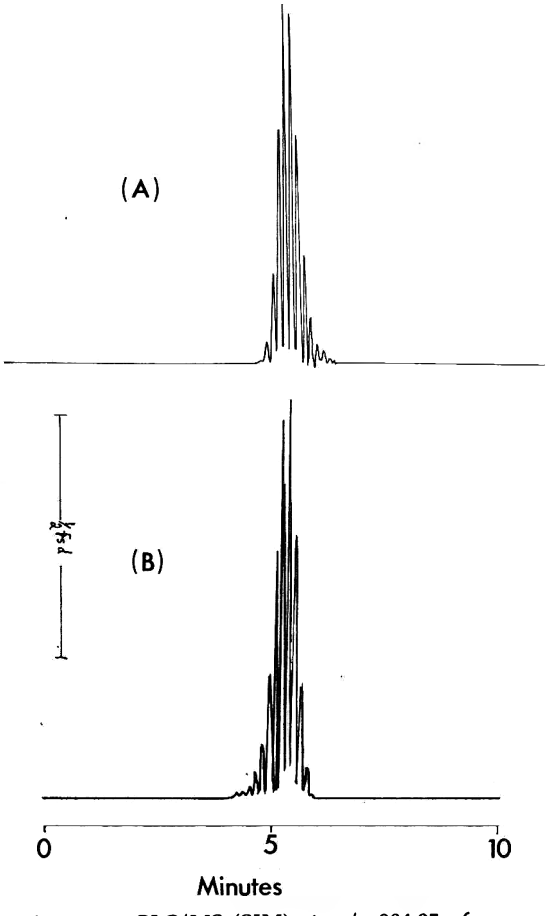


Figure 5. GLC/MS (SIM) at m/z 884.05 of same extract as Figure 4: A, sample containing 1.4 ppm; B, standard containing 5 ng vomitoxin tris-heptafluorobutyrate. Multiple peaks are a scan of the mass of interest.

In summary, we have developed a method for the detection of vomitoxin in cereal grains, which is sensitive to levels of 0.05 ppm. Samples were confirmed by GLC/MS using single ion monitoring at the molecular weight of the HFB ester (884.05). The capillary system with splitless injection gives the added advantage of extra resolution which was occasionally needed for difficult samples found to contain low levels of vomitoxin. The capillary system was reproducible, with a retention time of vomitoxin of 18.33 ± 0.03, calculated over a period of 3 days for 15 injections.

REFERENCES

(1) Rodricks, J. V., Hesseltine, C. W., & Mehlman, M. A. (1977) *Mycotoxins*, Pathotox Publishers, Park Forest South, IL, pp. 189–207, 256–261
(2) Romer, T. R. (1977) *Cereals Foods World* **22**, 521–523
(3) Jemmali, M., Uens, K., Ishii, K., Frayssinet, C., & Etienne, M. (1978) *Experientia* **10**, 1333–1334
(4) Muller, H. M. (1976) *Landwirtsch. Forsch. Sonderh.* **33**, 282–292
(5) Eppley, R. M. (1978) *J. Am. Oil. Chem. Soc.* **56**, 824–829
(6) Vesonder, R. A., Ceigler, A., Jensen, A., Rohwedder, W., & Weisledu, D. (1976) *Appl. Environ. Microbiol.* **31**, 280–284
(7) Mirocha, C. J., Pathre, C. V., Schauerhamer, B., & Christensen, C. M. (1976) *Appl. Environ. Microbiol.* **32**, 553–561
(8) Forsyth, D., Yoshizaura, T., Morooka, N., & Tuite, J. (1977) *Appl. Environ. Microbiol.* **34**, 347–351
(9) Romer, T. R., Boling, T. M., & MacDonald, J. L. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 801–808
(10) Mirocha, C. J., Pathre, V. S., & Behrens, J. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 221–223
(11) Collins, J. G., & Rosen, D. J. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 1274–1280

Determination and Thin Layer Chromatographic Confirmation of Identity of Aflatoxins B_1 and M_1 in Artificially Contaminated Beef Livers: Collaborative Study

ROBERT D. STUBBLEFIELD, WILLIAM F. KWOLEK, and LEONARD STOLOFF[1]
U.S. Department of Agriculture, Northern Regional Research Center, Agricultural Research Service, Peoria, IL 61604

Collaborators: J. M. Fremy; J. I. Greer; T. Juszkiewicz; W. Kronert; C. J. Meyer; D. S. P. Patterson; K. Pulzhofer; J. L. Richard; Y. Saito; P. L. Schuller; E. A. Sizoo; M. W. Trucksess; L. G. M. Th. Tuinstra; H. P. van Egmond; D. Wilson

An international collaborative study involving 13 laboratories was conducted to test methods for the determination and thin layer chromatographic (TLC) confirmation of identity of aflatoxins B_1 and M_1 in beef liver. For the determination, each collaborator furnished fresh or frozen beef liver. Samples were artificially contaminated by adding solutions containing various concentrations of aflatoxins B_1 and M_1 (0.032–0.69 ng/g). Two TLC confirmation methods were tested with extracts obtained from the determination. Two measurement methods using 2-dimensional TLC were evaluated. In the first, sample extracts were compared directly with B_1 and M_1 standards on TLC plates; in the second, internal standards plus sample extracts were compared with B_1 and M_1 standards on the plates. Average within-laboratory coefficients of variation (CV) for the direct method were 26% for B_1 and 26% for M_1 compared with 24 and 26%, respectively, for the internal standard method. The average between-laboratory CV values were 39% for B_1 and 41% for M_1 by the direct method and 36% for B_1 and 39% for M_1 by the internal standard method. Recoveries ranged from 64 to 90% for B_1 and from 72 to 86% for M_1. These data indicate that the more convenient direct method was sufficient, and internal standards were unnecessary. An analysis of variance was calculated from combined sample data to determine components of variance. The within-laboratory CV values were 27.0 and 32.3% for B_1 and M_1, respectively, and the between-laboratory CV values were 47.1 and 53.2%, respectively. Both TLC confirmation methods gave satisfactory results and have been adopted official first action, along with the determination method.

Aflatoxin-contaminated agricultural commodities (corn, peanuts, and cottonseed) have occurred worldwide. Observation of aflatoxin M_1 in milk indicates that contaminated commodities have been fed to dairy animals and probably to other farm animals. Therefore, it is important to examine the edible tissues of farm animals and poultry with current sensitive methods to see if aflatoxin residues in meats are a potential problem. Analytical methods for determining aflatoxins in tissue have improved in sensitivity and accuracy since 1976. A methods evaluation (unpublished) by mycotoxin analysts in France, The Netherlands, and the United States resulted in the method of Stubblefield and Shotwell (1) being tested in an international AOAC/IUPAC collaborative study along with thin layer chromatographic (TLC) confirmation method **26.A15** (2) and the method of van Egmond and Stubblefield (3).

Obtaining samples for this study was a major problem not normally encountered in collaborative studies. Sufficient naturally contaminated beef liver was not available to provide collaborators with identical, duplicate samples. Also of major importance was the cost and difficulty of shipping frozen livers to collaborators throughout the world. In 1980, L. Stoloff (Mycotoxin General Referee, AOAC) and M. Jemmali (Mycotoxin Working Group Chairman, IUPAC) undertook a joint collaborative study (unpublished) to test the methods with freeze-dried, naturally contaminated liver powder. The results of this study did not give the precision required for recommendation to AOAC or IUPAC. Part of the problem was due to sample preparation, because changes in the liver caused by freeze-drying makes sufficient cleanup of extracts very difficult. Fluorescent impurities remain in extracts for TLC and obscure the aflatoxin zones (1). To circumvent this problem, standard solutions of aflatoxins B_1 and M_1 were

This report of the Associate Referee, R. D. Stubblefield, was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, in Washington, DC.

[1] Food and Drug Administration, Washington, DC 20204.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Received August 12, 1981. Accepted June 28, 1982.

The recommendation of the Associate Referee was approved by the General Referee and Committee C and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* 65, 374 (1982).

0004-5756/82/6506-1435–10$01.00

provided to collaborators in the present study as samples, and collaborators provided fresh or frozen beef liver to be artificially contaminated. Restricting the study to fortified samples is not ideal, but it was a necessary compromise. We now report the study results.

Collaborative Study

Standard Aflatoxin B_1 and M_1 Solutions

Crystalline aflatoxins B_1 and M_1, with purity criteria previously reported (4, 5), were used to prepare stock solutions of 316.40 μg B_1/mL and 158.78 μg M_1/mL in acetonitrile. Concentrations were determined (in benzene–acetonitrile, 98 + 2 and 9 + 1, respectively) using extinction coefficients of 19 800 (B_1) and 18 815 (M_1) according to method **26.004-26.011** (2). Three standard solutions for TLC, containing 0.25 μg B_1, 0.25 μg M_1, and 0.25 μg B_1 + 0.25 μg M_1/mL in benzene–acetonitrile (9 + 1) were prepared and sealed in glass ampules.

Preparation of Solutions for Artificially Contaminating Beef Liver

Aliquots of B_1 (1.0 mL) and M_1 (1.0 mL) from stock solutions (see above) were added to 10 mL volumetric flasks to prepare 1:10 dilutions of stock B_1 (31.64 μg/mL) and stock M_1 (15.88 μg/mL) in acetone. Aliquots of the diluted stock solutions were added to 100 mL volumetric flasks to prepare the following sample solutions (acetone) for addition to uncontaminated and artificially contaminated samples: (a) 0.0 μg B_1 and 0.0 μg M_1/mL; (b) 3.2 ng B_1/mL; (c) 10.0 ng B_1 and 10.0 ng M_1/mL; (d) 30.0 ng B_1/mL; (e) 69.0 ng B_1 and 40.0 ng M_1/mL; (f) 40.0 ng B_1 and 20.0 ng M_1/mL; and (practice) 50.0 ng B_1 + 50.0 ng M_1/mL. Addition of 1.0 mL sample solution to 100 g blended liver gave aflatoxin concentrations of 0.00–0.69 ng/g. Sample solutions were dispensed in glass ampules, and ampules were sealed and randomly coded.

Methods

The method of Stubblefield and Shotwell (1) to determine B_1 and M_1 in animal tissue and the TFA–TLC confirmation method **26.A15** (overspot) (2) and van Egmond and Stubblefield method (3) (spray) were tested.

Description of Study

Sixteen collaborators each received the following items: 1 ampule each of the 3 standard solutions; 1 ampule of trifluoroacetic acid (TFA); 1 ampule of practice liver-contaminating solution; 12 ampules of liver-contaminating solutions (duplicates of spiking solutions numbered randomly); and a copy of the study instructions and report sheets. Each collaborator supplied fresh or frozen beef liver that was aflatoxin-free as determined by the quantitative method.

Aflatoxin concentrations were selected to test the methods at levels that might be encountered in edible meat tissues (0.03–0.7 ng/g). Collaborators were instructed to blend a sufficient quantity of uncontaminated beef liver until uniform, weigh 100 g into a 500 mL wide-mouth, glass-stopper Erlenmeyer flask, add 1 mL spiking solution, and mix thoroughly with a heavy glass rod in preparation for extraction. Collaborators were cautioned to prepare only the number of samples that they could analyze completely in 1 day. The practice spiking solution of stated concentration was included to familiarize analysts with the methods. Analysts were asked to use either or both TLC confirmatory methods and to return a photograph of each sample confirmatory plate with the report sheets. The collaborators were requested to quantitate each sample by the direct method (1) and by the internal standard (indirect) method. The latter consisted of overspotting the sample extract at the origin with 5 μL B_1 + M_1 standard solution and, after plate development and zone area measurement, deducting the internal standard. Both values were to be recorded on the report sheets. TLC plates with internal standard also served to locate B_1 and M_1 on the plates in relation to any interferences, and should be used for this purpose whether or not they are used for quantitation.

Aflatoxins B_1 and M_1 in Liver

Official First Action

26.C01 ***Apparatus***

(**a**) *Wrist-action shaker.*—Burrell, or equiv.

(**b**) *Meat grinder.*—Waring blender, Model EP-1, and any manual food grinder.

(**c**) *Chromatographic columns.*—Glass column 30 × 1.0 (id) cm with porous polyethylene frit (35 μm) and Luer nylon stopcock (Bio-Rad Econo-columns No. 737-2260 and 732-9009, resp., or equiv. glass column).

(**d**) *Filter paper.*—32 cm, S & S No. 588, or equiv. rapid flow, high wet-strength paper; and 24 cm, S & S No. 560, or Whatman 2V or equiv., medium flow paper.

(**e**) *Thin layer plates.*—10 × 10 cm com. prepoured, 0.25 mm thickness, glass plates (hand-cut

from 20 × 20 cm) (E. Merck silica gel 60, No. 5763 or Macherey, Nagel Sil G-25 HR), or prep. in laboratory as follows: 10 × 10 or 20 × 20 cm plates coated with 0.25–0.5 mm (wet thickness) layer of Macherey-Nagel GHR silica gel for TLC (Macherey, Nagel & Co., D-5160 Duren, GFR; distributed by Brinkmann Instruments, Inc.) dried 1 h at 105° or Adsorbosil-1 silica gel for TLC (Applied Science Laboratories, Inc.), or equiv.

26.C02 *Reagents*

(a) *Solvents.*—Reagent grade, distd in glass. Glacial HOAc, acetone, CH_3CN, benzene, $CHCl_3$ (0.75% EtOH), CH_2Cl_2, ether (≤0.1% EtOH, peroxide-free), hexane (68–69°), isopropanol, and toluene.

(b) *Citric acid soln.*—20%. Dissolve 200 g citric acid monohydrate in 1 L H_2O.

(c) *Silica gel for column chromatography.*—E. Merck silica gel 60 (No. 7734), 0.063–0.200 mm (70–230 mesh), or equiv. Stir 1 h in MeOH, filter, and treat similarly with $CHCl_3$. Activate by drying 1 hr at 105°. Add H_2O, 1 mL/100 g, seal, shake until thoroly mixed, and store ≥15 h in air-tight container.

(d) *Sodium sulfate.*—Anhyd., granular.

(e) *Diatomaceous earth.*—Hyflo Super-Gel.

(f) *Aflatoxin reference stds.*—Prep. as in **26.004–26.011** to contain 0.25 μg aflatoxin B_1 and M_1/mL in benzene–CH_3CN (9 + 1) for either visual or densitometric analysis. If aflatoxins G_1, B_2, and/or G_2 are needed, prep. G_1 at 0.25 μg/mL and B_2 and G_2 at 0.05 μg/mL. Store stds in 1 dram vials fitted with Teflon-lined screw caps at 0°F when not in use.

26.C03 *Extraction*

Blend or grind meat tissue until homogeneous. Weigh 100 g mixt. into 500 mL wide-mouth, g-s erlenmeyer (or equiv.). Add 10 mL citric acid soln and mix thoroly with 30 cm × 1 cm glass stirring rod. After 5 min, stir again, and mix with 20 g diat. earth. Add 200 mL CH_2Cl_2 and stir to remove excess solids from rod. Shake flask vigorously on wrist-action shaker (setting 5 on a Burrell) for 30 min. Filter mixt. thru fast flow paper into 300 mL erlenmeyer contg 10 g Na_2SO_4. Close filter top and compress entire filter against funnel to obtain max. filtrate vol. Gently swirl flask intermittently ca 2 min and refilter contents thru medium flow paper into 250 mL graduate and record vol. (cover funnel with watch glass to prevent evapn of solv). Evap. filtrate in 500 mL r-b flask, under vac., to near dryness and save for column chromatgy.

26.C04 *Column Chromatography*

Fill column half full with CH_2Cl_2 and add 2.0 g silica gel. Add 3–4 mL CH_2Cl_2 and slurry silica with stainless steel rod (ca 0.32 cm diam.). Drain CH_2Cl_2 to settle silica and rinse silica off column sides with CH_2Cl_2. Add 2 g Na_2SO_4 to supernate solv. above silica gel to cap column and drain excess CH_2Cl_2 to ca 1 cm above column packing.

Redissolve concd filtrate in ca 25 mL CH_2Cl_2, add to column, rinse r-b flask and column with addnl CH_2Cl_2, and drain entire soln thru column by gravity. If flow rate slows, stir Na_2SO_4 gently. When filtrate reaches Na_2SO_4, rinse column sides with CH_2Cl_2 and drain similarly. Wash column with 25 mL toluene–HOAc (9 + 1), 25 mL hexane, and 25 mL hexane–ether–CH_3CN (6 + 3 + 1) and discard washes. Elute aflatoxins with 40 mL CH_2Cl_2–acetone (4 + 1) and evap. eluate to near dryness in vac. or on steam bath. Quant. transfer ext with $CHCl_3$ or CH_2Cl_2 rinses to 1-dram vial with Teflon-lined screw cap. Evap. to dryness under N on heat source, but avoid overheating of dry ext. Save for TLC.

26.C05 *Visual and Densitometric Analysis*

Add 100 μL benzene–CH_3CN (9 + 1) to sample residue in vial from **26.C04**, cap vial, and mix vigorously ca 1 min, preferably on vortex mixer. After TLC analysis, reserve remaining ext in freezer for confirmation of identity.

See Figure **26:01** for spotting and scoring patterns of 2-dimensional TLC plates, except dimensions for 20 × 20 cm plate, direction 1, bottom to top are as follows: 2, 11, 1, 1, 1, 4 cm and dimensions for direction 2, left to right are 2, 12, 6 cm; similarly for 10 × 10 cm plate: direction 1: 1.5, 4.5, 1, 1, 1, 1 cm, and direction 2: 1.5, 6.5, 2 cm. Spot 20 μL aliquot of sample ext on sample spot and either 1.5, 0.5, 1.0, and 1.5 ng of ref. std (visual) or 2.5, 1.25, 1.25, and 2.5 ng of ref. (densitometric). Develop plate in ether–MeOH–H_2O (95 + 4 + 1) in first direction (see also **26.013**). When solv. reaches score line, remove plate, air-dry, heat in forced air oven at 50° (ca 2 min), cool plate, and redevelop in second direction in $CHCl_3$–acetone–isopropanol (87 + 10 + 3) to score line. Quantitate visually or densitometrically as in **26.074** and calc. concn of B_1 or M_1 as:

$$\mu g/kg = (S \times Y \times V)/(X \times W)$$

where S = μL aflatoxin ref. std equal to unknown; Y = concn of ref. std, μg/mL; V = μL of final diln of sample ext; X = μL sample ext spot-

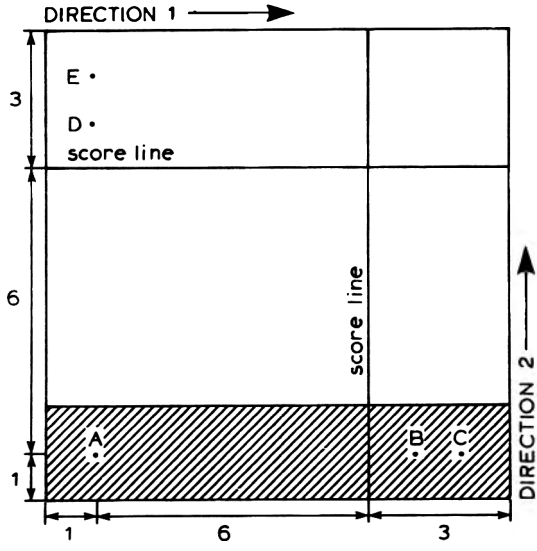


Figure 26:C1. Schematic representation of thin layer chromatogram for confirmation of identity of aflatoxins B_1 and M_1. A = spotting place for sample extract; B and D = spotting place for M_1 standard; C and E = spotting place for B_1 standard.

ted giving fluorescent intensity equal to *S* (ref. std); *W* = (100 g or mL × filtrate vol.)/200.

Confirmation of Aflatoxins B_1 and M_1 in Liver

Official First Action

26.C06 ***Reagents***

(a) *Solvents.*—$CHCl_3$ (≤0.75% EtOH), acetone, isopropanol, and hexane.

(b) *TFA-hexane spray.*—(1 + 4). Mix 1 vol. of trifluoroacetic acid (TFA, ≥95% pure) with 4 vols of hexane. Prep. fresh daily.

(c) *Aflatoxin std solns.*—Prep. sep. std solns of aflatoxins B_1 and M_1 (0.25 μg/mL each) in CH_3CN–benzene (1 + 9) or $CHCl_3$.

(d) *TLC plates.*—0.25 mm thick layer of Macherey-Nagel Sil-G-25HR silica gel (Macherey, Nagel & Co., D-5160, Duren, GFR, distributed by Brinkmann Instruments, Inc.) or Merck Kieselgel 60 on 10 × 10 cm plates, self-cut from 20 × 20 cm plates.

26.C07 ***Apparatus***

(a) *UV illumination cabinet.*—365 nm.

(b) *Disposable capillary pipets.*—10 and 20 μL, or microsyringes.

(c) *Spray unit for thin layer chromatography.*—Low vol. capacity (5–20 mL).

(d) *Air dryer.*—Unit capable of providing stream of warm air (40–50°) to evap. solv. from TLC plates.

26.C08 ***Thin Layer Chromatography***

Score 2 straight lines on 10 × 10 cm TLC plate at right angles (3 cm in from each edge) (see Figure 26:C1) to limit migration of developing solv. fronts. Spot following solns on plate, using capillary pipets or microsyringes:

(a) Vol. of sample ext equal to vol. used for quantitation on point A (normally ca 20 μL).

(b) Vol. of std soln contg ca 2.5 ng M_1 on points B and D.

(c) Vol. of std soln contg ca 2.5 ng B_1 on points C and E.

Develop plate in first direction with isopropanol–acetone–$CHCl_3$ ((3 + 10 + 87) for Macherey-Nagel TLC plates or (8 + 10 + 82) for Merck TLC plates) (see Figure 26:C1), until solv. front reaches solv. limit line. Dry plate after development 5–10 min with stream of warm air to evap. solv. completely (check odor). Spray TFA–hexane soln from a distance of 5–10 cm along band (ca 2 cm wide), covering points A, B, and C (indicated by hatched area in Figure 26:C1) until plate is thoroly sprayed (ca 2 mL spraying reagent). After hexane has evapd, cover TLC plate with warm, clean, glass plate (75°) and immediately heat 6–8 min in 75° oven with TLC plate on oven floor. Cool 1 min on cold surface, evap. excess TFA with stream of air, and develop in second direction with isopropanol–acetone–

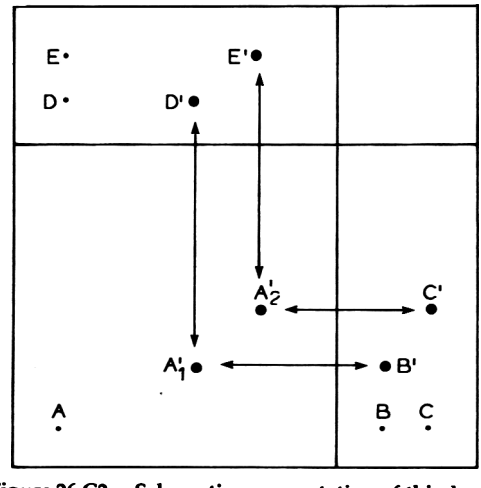


Figure 26:C2. Schematic representation of thin layer chromatogram after confirmation chromatography. A'_1 = M_1 derivative from sample; A'_2 = B_1 derivative from sample; B' = M_1 derivative from standard; C' = B_1 derivative from standard; D' = M_1 from standard; E' = B_1 from standard.

$CHCl_3$ ((6 + 10 + 84) for Macherey-Nagel TLC plates or (12 + 10 + 78) for Merck TLC plates).

Examine plate under longwave UV light (365 nm) and check for following fluorescent zones (see Figure 26:C2):

(a) Appearance of blue fluorescent spots D′ and E′ of std aflatoxins M_1 and B_1, resp., originating from D and E (migration in direction 1).

(b) Appearance of blue fluorescent spots B′ and C′ of TFA derivatives of aflatoxins M_1 and B_1, resp., originating from std solns spotted at B and C (migration in direction 2).

(c) Appearance of blue fluorescent spots A_1' and/or A_2' originating from ext spotted at A, with R_f values matching those of spots B′ and C′, resp.

Identity of aflatoxin B_1 in ext is confirmed when R_f values of B_1 derivative from Sample (A_2') and B_1 std (C′) match. Similarly, identity of aflatoxin M_1 in ext is confirmed when R_f values of M_1 derivative from Sample (A_1') and M_1 std (B′) match.

Results

Individual values were omitted from calculations according to Dixon's test for outliers at the 0.05 level (6). For statistical calculations, the second value submitted was substituted for the outlier to maintain balance in the analysis of variance. Similar treatment was given to spilled Sample 5 for Collaborator 14. Values for Collaborators 2 and 16 were not included in the calculations because the composite data for each exceeded the lower limit of Youden's ranking test (7). Collaborators 7 and 13 were borderline in the same test, but both are included in the calculations. Collaborators 8 and 10 did not submit results by both the indirect and direct measurement procedures, so their data were not used in analysis of variance calculations.

The results reported for aflatoxins B_1 and M_1 in artificially contaminated beef liver are given in Table 1. The statistical summary for these results is presented in Table 2. Means for both B_1 and M_1 obtained by the internal standard method were lower than those from the direct method. However, there is no significant statistical difference between them. Although a TLC plate with an internal standard should always be prepared to establish the chromatographic separation of the aflatoxins from interferences, quantitation of this plate does not appear to improve accuracy and precision of the total measurement. Recoveries for aflatoxin B_1 by the direct method were 64–90%. The statistical data (% recovery) for Sample 4 strongly suggest that an error occurred in sample solution preparation by the Associate Referee. Based on the other recoveries, this sample probably contained 0.22–0.23 ng B_1/g instead of the intended 0.30 ng/g. Recoveries for aflatoxin M_1 by the direct method ranged from 72 to 86%. The low value was found at the 0.10 ng/g level, the lowest level tested. Coefficients of variation (CV) for B_1 and M_1 were 31–54% by the direct method. These CV values are comparable to those calculated in other aflatoxin collaborative studies. Of the 352 determinations (including Collaborators 8 and 10), there were 7 false negatives for B_1 and 2 false negatives for M_1, all at the lowest levels tested (0.03 ng B_1/g and 0.10 ng M_1/g) (Table 1); most of these were from plates for which subtraction of the internal standard resulted in a small negative value. If only the results obtained by direct measurement are used, only 2 false negatives (B_1, Sample 2, Collaborator 10) were reported.

The results for uncontaminated beef liver are given in Table 3. There were 6 aflatoxin B_1 false positives for 42 total observations, of which only 2 were by direct determination. The identity of 1 of these was not confirmed. Of the 130 M_1 observations, there were 16 false positives. Only 4 (0.1 ng/g) were reported by the direct method; however, the identities of all 4 were confirmed. Most of the fluorescent contaminants present in the liver extracts occur in the M_1 area of the TLC plate. Either those collaborators with false positives accidentally contaminated their samples or, if not, they should experiment with solvent systems to better resolve M_1 from contaminants. Also, livers used for spiking could have been contaminated if not checked beforehand. Obvious large differences in TLC interferences were observed based on the photographs. This was expected because of the different animal breeds, ages, gender, and feed rations involved for the beef livers. Although these factors prevented identical samples from being tested, they did provide for realistic samples as would be encountered in practice, except that all were artificially contaminated.

The precision estimates calculated to compare measurement methods for B_1 and M_1 on an individual sample basis are given in Table 4. The within-laboratory coefficient of variation (CV_o) is the repeatability, and the between-laboratory coefficient of variation (CV_x) is the reproducibility. Averages for B_1 were 26% (CV_o) and 39% (CV_x) by the direct method, with very little difference (24 and 36%, respectively) by the internal standard method. The averages for aflatoxin M_1

Table 1. Collaborative study results (ng/g × 10^2) for determination of aflatoxins B_1 and M_1 in artificially contaminated beef liver [a]

		Sample 2		Sample 3				Sample 4		Sample 5				Sample 6			
Coll.	Meth[b]	B_1		B_1		M_1		B_1		B_1		M_1		B_1		M_1	
1	I	1.7	1.8	9.6	5.9	3.4	2.9	20.8	28.7	65.3	70.6	28.0	20.0	33.1	37.9	9.5	9.5
	D	2.2	2.4	8.7	5.9	3.4	2.9	25.0	32.8	70.0	70.6	28.0	25.2	35.5	37.9	11.8	11.8
2[c]	D	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(10.0)	(29.0)	(24.0)	(22.0)	(17.0)	(0)	(11.0)	(0)	(7.0)
3	I	2.0	2.0	7.0	8.0	3.0	7.0	24.0	23.0	60.0	89.0	28.0	41.0	28.0	46.0	14.0	19.0
	D	3.0	2.0	10.0	8.0	6.0	10.0	23.0	21.0	61.0	91.0	29.0	45.0	31.0	44.0	14.0	20.0
4	I	3.0	2.0	8.0	7.0	5.0	7.0	17.0	22.0	51.0	83.0	25.0	17.0	49.0	48.0	10.0	19.0
	D	3.0	2.0	8.0	9.0	5.0	5.0	17.0	22.0	59.0	100.0	25.0	25.0	49.0	32.0	10.0	19.0
5	I	1.4	0.4	5.0	5.6	5.9	4.9	29.0	19.0	75.0	57.0	40.0	25.0	42.0	35.0	16.0	15.0
	D	1.7	1.6	6.3	5.2	7.3	7.8	29.0	19.0	83.0	61.0	41.0	29.0	40.0	41.0	17.0	17.0
6	I	2.5	4.0	8.0	10.0	7.0	8.0	25.0	24.0	81.0	102.0	51.0	50.0	40.0	48.0	21.0	27.0
	D	2.5	4.0	8.0	10.0	7.0	8.0	25.0	24.0	81.0	102.0	51.0	50.0	40.0	48.0	21.0	27.0
7	I	3.7	2.0	6.0	3.6	2.5	6.5	11.0	10.1	46.5	31.2	28.5	21.6	30.4	20.2	6.5	12.2
	D	4.4	5.6	0	3.4	1.8	4.0	12.0	13.7	44.5	25.8	26.2	20.3	30.1	21.4	7.7	9.3
8[d]	I	0	(3.0)[e]	(3.0)[e]	(3.0)[e]	0	0	20.0	3.3	30.0	30.0	7.0	5.0	8.0	7.0	8.0	3.0
10[d]	D	0	0	5.0	5.0	15.0	13.0	9.0	13.0	20.0	60.0	34.0	70.0	9.0	9.0	21.0	21.0
11	I	5.0	8.0	10.0	13.0	4.0	13.0	23.0	20.0	89.0	89.0	53.0	50.0	44.0	33.0	20.0	26.0
	D	4.0	4.0	9.0	10.0	6.0	10.0	26.0	26.0	77.0	82.0	35.0	48.0	48.0	33.0	22.0	15.0
13	I	0	0	6.0	3.0	2.0	1.0	8.0	7.0	26.0	54.0	20.0	22.0	31.0	23.0	7.0	8.0
	D	1.0	(3.0)[e]	7.0	8.0	4.0	6.0	9.0	7.0	32.0	56.0	25.0	26.0	32.0	27.0	12.0	11.0
14	I	1.2	3.3	5.0	5.4	5.5	10.5	12.5	12.5	42.0	(27.3)[f]	23.1	(17.3)[f]	18.1	16.0	16.1	14.7
	D	2.2	4.9	8.6	4.2	8.5	12.4	13.8	17.1	54.6	(21.8)[f]	34.3	(14.1)[f]	28.9	19.7	14.2	14.0
15	I	(11.0)[g]	(3.0)[e]	11.0	8.0	17.0	9.0	10.0	26.0	20.0	33.0	17.0	30.0	38.0	31.0	19.0	22.0
	D	(11.0)[g]	(3.0)[e]	12.0	7.0	(19.0)[g]	9.0	11.0	25.0	29.0	39.0	22.0	31.0	31.0	26.0	19.0	22.0
16[c]	D	(0)	(0)	(0)	(0)	(3.0)[e]	(10.0)	(3.0)[e]	(3.0)[e]	(3.0)[e]	(10.0)	(35.0)	(15.0)	(0)	(0)	(10.0)	(0)

[a] As determined by the method of Stubblefield and Shotwell (1).
[b] Method of measurement: (D) = sample zones directly compared with standards; (I) = internal standard spotted with sample extract and compared with standards.
[c] Values omitted from calculations after applying Youden's ranking test (7).
[d] Collaborator submitted either direct or indirect data only. Values not included in analysis of variance.
[e] Collaborator reported trace. Trace was taken as 0.03 ng/g for statistical purposes.
[f] Sample was spilled. Values not used in calculations.
[g] Values omitted from calculations as outliers by Dixon's test (6).

Table 2. Collaborative study statistical results for determination of aflatoxins B_1 and M_1 in artificially contaminated beef liver [a]

	Sample 2	Sample 3		Sample 4	Sample 5		Sample 6	
Statistic	B_1	B_1	M_1	B_1	B_1	M_1	B_1	M_1
Direct measurement								
Mean, ng/g	2.69	7.20	7.24	19.11	6[illegible].83	34.29	32.43	16.22
SD	1.464	2.685	3.462	7.257	23.931	12.406	11.118	5.077
CV, %	54.41	37.31	47.80	37.98	38.70	36.19	34.28	31.30
Theor., ng/g	3.2	10.0	10.0	30.0	69.0	40.0	40.0	20.0
Rec., %	84.1	72.0	72.4	63.7	89.6	85.7	81.1	81.1
N	21	22	21	22	21	21	22	22
Internal standard measurement								
Mean, ng/g	2.54	7.03	6.45	18.55	60.07	31.48	32.28	15.44
SD	1.662	2.677	3.864	7.309	24.140	13.341	11.688	5.834
CV, %	65.57	38.07	59.94	39.39	40.18	42.38	36.21	37.79
Theor., ng/g	3.2	10.0	10.0	30.0	69.0	40.0	40.0	20.00
Rec., %	79.4	70.3	64.5	61.8	87.1	78.7	80.7	77.2
N	42	44	43	44	42	42	44	44

[a] Calculated from values given in Table 1. Means, standard deviations, and theoretical values are actual values × 10^2.

were 26% (CV_o) and 41% (CV_x) (direct) and 26% (CV_o) and 39% (CV_x) (internal standard). From these data and those in Table 2, neither accuracy nor precision is increased by including a measurement of the TLC plate with internal standard, and a definite possibility exists of misinterpreting a measurement as either false positive or false negative.

A more complete understanding of the total variance and the component sources of variation that comprise it is obtained by determining an analysis of variance for the entire composite of samples. A larger number of observations are available for variation estimates, which tend to yield more reliable statistical results. The analysis of variance and variance components after combining data for Samples 3–6 are given in Table 5. Data for these samples were computed by using the values reported from the 10 collaborators that submitted both direct and indirect results. A log transformation was used to compute the analysis of variance as recommended by Snedecor and Cochran (8) when standard deviations (Table 2) are proportional to the means rather than constant. This is true in this study because the CV values are relatively constant, although sample means vary by a factor of 4–6. As noted earlier, Collaborators 7 and 13 were associated with significant rank sums and could have been omitted from the overall analysis. However, their data were retained and tend to inflate the laboratory and laboratory × concentration variances, but with little effect on the sample-replicate and remainder variation (D + S + I). The between-laboratory variance ratios (*F*-test: 7.65 for B_1, 7.48 for M_1) (not shown) are significant, whereas the concentration–laboratory interaction variance ratio is significant for B_1 but not for M_1. The laboratory biases are not surprising, considering the differences in beef livers discussed earlier. The coefficients of variation calculated from the various components of variance (Table 5, bottom) are consistent with other aflatoxin collaborative studies that tested low-level samples. The within-laboratory CV value (repeatability) for B_1 was 27%, and for M_1, 32%. The between-laboratory CV values (reproducibility) were 47 and 53%, respectively. The analysis of variance also indicates significant variation associated with methods in the case of B_1. The direct method for Samples 3–6 tends to yield a somewhat higher result (Table 2).

The results for the TLC confirmation of identity are given in Table 6. For contaminated samples, 12 false negatives were reported for 120 B_1 observations and 2 false negatives were reported for 72 M_1 observations. Eight of the 12 B_1 false negatives were at the lowest level (0.03 ng/g) and 1 false negative was at 0.10 ng/g. Both M_1 false negatives were at the lowest M_1 level—0.10 ng/g. It is not apparent from the data whether the overspot method (2) or the spray method (3) is more dependable, because the false negatives were about equally divided between the 2 methods. For the uncontaminated samples (Table 6), 2 false positives were reported for 23 B_1 observations, and 6 false positives were reported for 71 M_1 observations. Similarly, no clear distinction existed between methods for quantitation. With either method, the liver interferences present on the plates can cause difficulties, especially for those not expe-

Table 3. Collaborative study results (ng/g × 10^2) for determination of aflatoxins B_1 and M_1 in uncontaminated beef liver [a]

		Sample 1				Sample 2		Sample 4	
Coll.	Meth[b]	B_1		M_1		M_1		M_1	
1	I	0	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0	0
2[c]	D	(0)	(0)	(0)	(3.0)[d]	(0)	(3.0)[d]	(0)	(3.0)[d]
3	I	0	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0	0
4	I	0	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0	0
5	I	0	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0	0
6	I	0	0	0	0	1.0	22.0	0	0
	D	0	6.0	0	0	1.0	22.0	0	0
7	I	1.0	0	0	5.2	4.2	0.8	1.8	0
	D	0	0	0	0	0.8	0	0	0
8	I	(3.0)[d]	0	0	0	0	0	8.0	0
10	D	0	0	0	0	0	0	0	0
11	I	6.0	3.0	0	3.0	3.0	5.0	0	3.0
	D	9.0	0	0	0	0	0	0	0
13	I	(—)[e]	0	(—)[e]	0	0	0	0	0
	D	(—)[e]	0	(—)[e]	0	0	0	0	0
14	I	0	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0	0
15	I	0	0	0	0	20.0	0	0	0
	D	0	0	0	0	19.0	0	0	0
16[c]	D	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
				Total Observations					
Internal std		21		21		22		22	
Direct		21		21		22		22	
				Positive Observations					
Internal std		4		2		7		3	
Direct		2		0		4		0	
				Negative Observations					
Internal std		17		19		15		19	
Direct		19		21		18		22	

[a] As determined by the method of Stubblefield and Shotwell (1).
[b] Method of measurement: (D) = direct comparison with standards; (I) = internal standard spotted with sample extract and compared with standards.
[c] Values omitted from calculations after applying Youden's ranking test (7).
[d] Collaborator reported trace. Trace was taken as 0.03 ng/g for calculations.
[e] Ampule broke.

Table 4. Precision estimates for measurement methods to determine aflatoxins B_1 and M_1 in artificially contaminated beef liver [a]

	Aflatoxin B_1					Aflatoxin M_1			
		Direct		Internal std			Direct		Internal std	
Sample	Toxin added, ng/g	CV_o	CV_x	CV_o	CV_x	Toxin added, ng/g	CV_o	CV_x	CV_o	CV_x
2	0.032	30	44	30	47	—				
3	0.10	26	38	20	29	0.10	35	55	37	45
4	0.30	23	39	24	37	—				
5	0.60	28	39	24	38	0.40	19	30	19	35
6	0.40	20	35	18	26	0.20	21	33	19	35
Av.		26	39	24	36		26	41	26	39

[a] Calculated for values in Table 1. CV_o is within-laboratory coefficient of variation (repeatability); CV_x is between-laboratory coefficient of variation (reproducibility). CV values are percentages.

Table 5. Analysis of variance for combined samples [a]

Variation source [b]	Aflatoxin B_1 DF [c]	Aflatoxin B_1 Mean square	Aflatoxin M_1 DF [c]	Aflatoxin M_1 Mean square	Expected mean square
Laboratories (L)	9	0.2526**	9	0.2407**	$\sigma^2 + 2\sigma_o^2 + 4\sigma_{Lc}^2 + \sigma_L^2$
Concentrations (C)	3	6.1426**	2	5.4698**	
Lab. × concn interaction (I)	27	0.0330*	18	0.0322	$\sigma^2 + 2\sigma_o^2 + 4\sigma_{Lc}^2$
Samples–replicates (S)	40	0.0185**	30	0.0259**	$\sigma^2 + 2\sigma_o^2$
Method (M)	1	0.0240**	1	0.0641**	
M × L	9	0.0067	9	0.0282**	
M × C	3	0.0012	2	0.0111	
M × I	27	0.0045	18	0.0111	
Duplicates (D)	38	0.0032	26	0.0057	σ^2
		Coefficients of Variation, % [d]			
Duplicates (σ^2)		13.9		19.0	
D + samples ($\sigma^2 + \sigma_o^2$) (Within-lab. repeatability)		27.0		32.3	
D + S + interaction ($\sigma^2 + \sigma_o^2 + \sigma_{Lc}^2$)		31.8		34.9	
D + S + I + lab. ($\sigma^2 + \sigma_o^2 + \sigma_{Lc}^2 + \sigma_L^2$) (Between-lab. reproducibility)		47.1		53.2	

[a] Analysis of variance calculated by log transformation according to Snedecor and Cochran (8). Values are logarithms.
[b] Laboratories = collaborators, method = direct vs indirect, samples = replicates.
[c] DF = degrees of freedom.
[d] Calculated from anti-logarithm formula: CV = $(10^S - 1) \times 100$, where S = σ^2 ($\sigma^2 + \sigma_o^2$, $\sigma^2 + \sigma_o^2 + \sigma_{Lc}^2$, or $\sigma^2 + \sigma_o^2 + \sigma_{Lc}^2 + \sigma_L^2$).
** = significant variation at 0.01 level; * = significant variation at 0.05 level.

Table 6. Summary of collaborative study results for TLC confirmation of identity of aflatoxins B_1 and M_1 in beef liver [a]

Artificially contaminated								Uncontaminated			
Sample 2	Sample 3		Sample 4	Sample 5		Sample 6		Sample 1		Sample 2	Sample 3
B_1	B_1	M_1	B_1	B_1	M_1	B_1	M_1	B_1	M_1	M_1	M_1
Total Observations											
24	24	24	24	24	24	24	24	23	23	24	24
Positive Observations											
16	21	22	24	24	24	23	24	2	3	4	2
Negative Observations											
8	3	2	0	0	0	1	0	21	23	20	22

[a] As determined by the TFA–TLC method **26.A15** (2) (overspot) or van Egmond and Stubblefield (3) (spray). Collaborator 2 did not report results. Collaborator 16 data not included because quantitative data exceeded Youden's ranking test (7), and Collaborator 13's Sample 1 ampule broke. Four collaborators used overspot method only; 3 collaborators used spray method only; 4 collaborators used both methods; and 1 collaborator did not specify which method was used.

rienced in analyzing liver tissues. Also, the B_1 and M_1 zones from the sample spot developed 2-dimensionally tend to have lower R_f values than the standards developed in one dimension (1), because more sample extract is spotted on a plate than for other aflatoxin methods. Logically, one would use the overspot method to reduce the number of plates needed. The spray method is a good alternative TLC confirmation of identity. It is recommended that the determination and both confirmatory methods be adopted official first action.

Acknowledgments

We thank R. Albert, FDA, Washington, DC, for the individual sample precision estimates, and the following collaborators for their cooperation:

J. M. Fremy, Laboratoire Central d'Hygiene Alimentaire, Paris, France

J. I. Greer, Northern Regional Research Center, USDA, Peoria, IL

T. Juszkiewicz, Veterinary Research Institute, Pulway, Poland

W. Kronert, Max von Pettenkofer Institut des Bundesgesundheitsamtes, Berlin, GFR

C. J. Meyer, National Research Institute for Nutritional Diseases, Tygerberg, South Africa

D. S. P. Patterson, Central Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food, New Haw Weybridge, Surrey, UK

K. Pulzhofer, Unilever Research Laboratory, Hamburg, GFR

J. L. Richard, National Animal Disease Center, USDA, Ames, IA

Y. Saito, National Institute of Hygienic Sciences, Division of Foods, Tokyo, Japan

P. L. Schuller, E. A. Sizoo, and H. P. van Egmond, National Institute of Public Health, Bilthoven, The Netherlands

M. W. Trucksess, Food and Drug Administration, Washington, DC

L. G. M. Th. Tuinstra, Government Dairy Station, Leiden, The Netherlands

D. Wilson, University of Georgia, Tifton, GA

REFERENCES

(1) Stubblefield, R. D., & Shotwell, O. L. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 964–968

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

(3) van Egmond, H. P., & Stubblefield, R. D. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 152–155

(4) Stubblefield, R. D., Shotwell, O. L., & Shannon, G. M. (1968) *J. Am. Oil. Chem. Soc.* **45**, 686–688

(5) Stubblefield, R. D., Shannon, G. M., & Shotwell, O. L. (1971) *J. Am. Oil Chem. Soc.* **47**, 389–390

(6) Dixon, W. J., & Massey, F. J. (1957) *Introduction to Statistical Analysis*, 2nd Ed., McGraw-Hill Book Co., New York, NY, p. 277

(7) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA

(8) Snedecor, G. W., & Cochran, W. C. (1980) *Statistical Methods*, 7th Ed., Iowa State University Press, Ames, IA, pp. 291–292

DRUGS

Spectrophotometric and Titrimetric Determination of Certain Adrenergic Drugs, Using Organic Brominating Agents

MOHAMED I. WALASH, A. ABOU OUF, and FATMA B. SALEM
Mansoura University, Chemistry Department, Faculty of Pharmacy, Mansoura, Egypt

New oxidimetric titrants, bromamine T, dibromohydantoin, *N*-bromophthalimide, and *N*-bromosuccinimide, were applied to the determination of ephedrine.HCl, norephedrine.HCl, and methyldopa. Direct potentiometric and visual indicator titration methods as well as back-titration procedures have been developed for their determination. Oxidation of ephedrine and norephedrine produces benzaldehyde, which is extracted from pH 11.0 phosphate buffer with ether or hexane and determined spectrophotometrically at 242 nm. Beer's law is obeyed in the concentration range from 0.2 to 2 mg ephedrine.HCl and from 0.15 to 1.9 mg norephedrine.HCl. Methyldopa is determined titrimetrically and spectrophotometrically. In addition, this drug acts as a self-indicator: Solutions change from colorless to red, which gradually disappears with continuous addition of brominating agent and shaking. Phosphate buffer is used to produce adrenochrome, characterized by its pink color which can be measured at 485 nm in a working range from 40 to 650 μg.

The utility of ephedrine, phenylpropanolamine, and other compounds which may be considered phenethanolamine derivatives has led to the publication of many methods for their determination in pharmaceutical dosage forms. A review of the literature (1–3) showed that a variety of methods have been used to quantitatively determine these active ingredients in pharmaceutical formulations. The methods used included diatomaceous earth separation followed by spectrophotometric procedures, gas-liquid chromatography, anion-exchange chromatography followed by titration with hydrochloric acid, and periodate oxidation to benzaldehyde.

The variety of methods used for the determination of methyldopa (4, 5) have been based on the measurement of color produced by reaction with iron salts in an alkaline medium, and spectrophotometric, titrimetric, fluorometric, and colorimetric procedures. Abou Ouf et al. (6) published a method for determining catecholamines, using brominating agents. The method proposed here is based on the oxidation of methyldopa, ephedrine, and norephedrine with organic brominating agents, and the spectrophotometric and titrimetric determination of the end products. These procedures are applied to pharmaceutical dosage forms available in Egypt.

Received February 9, 1982. Accepted March 23, 1982.

Experimental

Materials

(a) *Apparatus.*—Unicam SP 1800 spectrophotometer. Unicam Model 292 pH meter, glass electrode No. 529560, and platinum and calomel electrodes Nos. 527653 and 529571.

(b) *Buffer solutions.*—Phosphate buffer solution pH 7.4: 50C mL 0.1M potassium dihydrogen orthophosphate solution and 390 mL 0.1N NaOH were mixed and diluted to 1 L with distilled water. Phosphate buffer solution pH 11.0: 500 mL 0.05M disodium hydrogen phosphate solution and 40 mL C.1M NaOH solution were mixed and diluted to 1 L with distilled water.

(c) *Bromamine T (BAT).*—Prepared according to Nair et al. (7). Approximately 0.02M solution was prepared by dissolving 6.53 g BAT in 1 L water, kept in amber-colored bottles, and standardized iodimetrically.

(d) *N-Bromosuccinimide (NBS).*—Freshly recrystallized. A 0.02M solution was prepared by dissolving 3.56 g in minimum volume of water and diluting to 1 L with water. Solution was freshly prepared before use and standardized iodimetrically (8).

(e) *N-Bromophthalimide (NBP).*—Freshly recrystallized. A 0.02M solution was prepared by dissolving 4.52 g in minimum volume of glacial acetic acid and diluting to 1 L with glacial acetic acid. Solution was freshly prepared before use and standardized iodimetrically (9).

(f) *Dibromohydantoin (DBH).*—Synthesized according to Burry et al. (10) and recrystallized from hot water. A 0.02M solution was prepared by dissolving 5.72 g in hot water, cooling, and

Table 1. Results for titrimetric determination of ephedrine.HCl, norephedrine.HCl, and methyldopa [a]

Drug	Range, mg	Recovery with NBS, %				Recovery with DBH, %			
		Titrn with indicator	Titrn without indicator	Potentiometric	Back titrn	Titrn with indicator	Titrn without indicator	Potentiometric	Back titrn
Ephedrine.HCl	4.04–60.6	99.32 ± 0.283	—	99.05 ± 2.0	99.16 ± 0.16	99.472 ± 0.253	—	99.31 ± 0.173	99.34 ± 0.202
t [b]		3.36	—	1.471	3.48	4.4	—	3.7	4.23
F [c]		1.51	—	1.92	1.01	1.3	—	1.732	3.419
Norephedrine.HCl	3.942–56.13	98.959 ± 0.46	—	98.84 ± 0.53	99.03 ± 0.433	98.95 ± 0.46	—	99.01 ± 0.44	99.264 ± 0.342
t		0.27	—	0.273	0.71	0.261	—	0.595	2.42
F		2.34	—	3.896	1.1	2.64	—	2.31	1.642
Methyldopa	4.22–33.76	99.13 ± 0.39	100.72 ± 0.76	99.18 ± 0.59	99.25 ± 0.32	99.34 ± 0.34	100.51 ± 0.721	99.38 ± 0.33	99.452 ± 0.37
t		2.26	6.36	1.803	3.370	3.81	5.93	1.803	4.24
F		1.783	15.36	3.73	17.17	1.54	4.17	1.136	5.032

[a] Each result is mean of 6 experiments ± 95% confidence interval. Recoveries by official method: ephedrine.HCl = 98.9 ± 0.164; norephedrine.HCl = 98.9 ± 0.64; methyldopa = 98.731 ± 0.218%.

diluting to 1 L with water. Solution was freshly prepared before use and standardized iodimetrically.

(g) *Indicator solutions.*—Aqueous solutions (0.1%) of amaranth, methyl red, methyl orange, and indigotine were prepared and stored in amber bottles.

(h) *Active ingredients and dosage forms.*—Sympathomimetic amines used were ephedrine.HCl, norephedrine.HCl, and methyldopa. Purities were established according to the *U.S. Pharmacopeia* (11). All compounds had a purity ≥98.5% active ingredients. Dosage forms were analyzed for these contents.

Stoichiometric Study

The quantitative nature of the reaction between ephedrine, norephedrine.HCl, and the brominating agents was checked by titrating a 0.02M solution with the reagent solutions. The molar ratio was calculated to be 1:1 with BAT, NBS, NBP and 2:1 with DBH in acid medium, and 1:2 with BAT, NBS, NBP and 1:1 with DBH in pH 11.0 phosphate buffer. For methyldopa, the stoichiometry was calculated to be 1:3 with BAT, NBS, NBP and 2:3 with DBH. In titration without indicator, the molar ratio was 1:6 with BAT, NBS, NBP and 1:3 with DBH; this ratio was the same in pH 7.4 phosphate buffer.

Table 2. Results for titrimetric determination of ephedrine, norephedrine, and methyldopa in pharmaceutical preparations [a]

Sample	Active ingredient	Recovery with NBS, %				Recovery with DBH, %			
		Titrn with indicator	Titrn without indicator	Potentiometric	Back-titrn	Titrn with indicator	Titrn without indicator	Potentiometric	Back-titrn
Ephedrine tablets [b]	ephedrine.HCl	99.44 ± 0.32	—	99.59 ± 0.29	99.23 ± 0.29	99.5 ± 0.28	—	99.5 ± 0.47	99.25 ± 0.34
Ephedrine ampules [c]	ephedrine.HCl	99.34 ± 0.14	—	99.0 ± 0.46	99.195 ± 0.221	99.384 ± 0.103	—	99.09 ± 0.35	99.264 ± 0.26
Asmacid tablets [d]	ephedrine.HCl	99.382 ± 0.42	—	99.422 ± 0.45	99.29 ± 0.24	99.384 ± 0.41	—	99.45 ± 0.47	99.3 ± 0.24
Tussipect syrup [e]	ephedrine.HCl	99.382 ± 0.28	—	99.32 ± 0.22	99.26 ± 0.453	99.34 ± 0.19	—	99.24 ± 0.25	99.29 ± 0.4
Noflu tablets [f]	phenylpropanolamine.HCl	99.2 ± 0.19	—	99.25 ± 0.51	99.37 ± 0.262	99.164 ± 0.17	—	99.44 ± 0.43	99.44 ± 0.212
Aldomet tablets [g]	methyldopa	98.99 ± 0.52	101.78 ± 0.96	99.1 ± 0.48	99.03 ± 0.31	99.03 ± 0.53	101.74 ± 0.89	99.26 ± 0.35	99.19 ± 0.252

[a] Each result is the mean of 6 experiments ± 95% confidence interval.
[b] Labeled to contain 30 mg per tablet (Misr, Egypt).
[c] Labeled to contain 30 mg per mL (Misr, Egypt).
[d] Labeled to contain 15 mg ephedrine.HCl, 120 mg theophylline, 25 mg meclizine.HCl, 10 mg phenobarbitone per tablet (Cid, Egypt).

Table 1 (continued)

Recovery with NBP, %				Recovery with BAT, %			
Titrn with indicator	Titrn without indicator	Potentiometric	Back titrn	Titrn with indicator	Titrn without indicator	Potentiometric	Back titrn
98.97 ± 0.025	—	98.73 ± 0.55	98.97 ± 0.613	99.36 ± 0.18	—	99.28 ± 0.161	99.32 ± 0.14
1.04	—	1.14	0.98	4.6	—	4.09	4.884
1.94	—	1.88	1.21	2.71	—	1.652	3.41
98.652 ± 0.72	—	97.86 ± 0.93	98.54 ± 0.46	99.142 ± 0.05	—	99.4 ± 0.53	99.282 ± 0.403
0.85	—	2.77	1.85	1.72	—	2.273	2.21
3.47	—	6.986	5.71	2.374	—	4.34	7.1
98.56 ± 0.7	100.97 ± 0.8	98.4 ± 0.6	98.682 ± 0.7	99.15 ± 0.38	100.47 ± 0.16	99.32 ± 0.33	99.452 ± 0.36
0.592	7.0	1.31	0.17	2.44	0.872	3.682	4.35
9.73	15.34	5.03	4.17	1.651	24.32	1.483	2.36

[b] Tabulated t (10 DF) = 2.23 at 0.05 significance level.
[c] F (5.5 DF) = 5.1 at 0.05 significance level.

Procedure A: Pure Drugs, Titrimetric Method

An accurately measured volume of solution containing 4.0–60 mg ephedrine.HCl, 3.0–55 mg norephedrine.HCl, and 4.0–35.0 mg methyldopa was transferred to a 100 mL beaker, 10 mL concentrated HCl was added, and the solution was diluted to 50 mL with water. The solution was titrated with a standard solution of brominating agent with the aid of a magnetic stirrer; the end point was determined by using 3 drops of any indicator solution until color of the solution was discharged.

The end point could be determined for methyldopa without indicator. During addition of titrant, the solution changed from colorless to red, which gradually disappeared on continuous addition of brominating titrant and shaking. Titration was carried out in 0.01N HCl.

Alternatively, titration could be followed potentiometrically by using a combination of platinum and calomel electrodes.

Also, known volumes of solutions containing 4.0–60 mg ephedrine.HCl, 3.0–355.0 mg norephedrine.HCl, and 4.0–35.0 mg methyldopa were added to a measured excess of organic brominating solution in an iodine flask and the mixture was kept at room temperature with occa-

Table 2 (continued)

Recovery with NBP, %				Recovery with BAT, %			
Titrn with indicator	Titrn without indicator	Potentiometric	Back-titrn	Titrn with indicator	Titrn without indicator	Potentiometric	Back-titrn
101.39 ± 0.94	—	101.25 ± 0.953	99.26 ± 0.34	99.62 ± 0.59	—	99.51 ± 0.4	99.23 ± 0.32
100.93 ± 0.5	—	98.21 ± 0.57	99.132 ± 0.26	99.39 ± 0.08	—	99.09 ± 0.35	99.25 ± 0.243
101.45 ± 0.7	—	101.04 ± 1.21	99.264 ± 0.323	99.39 ± 0.37	—	99.61 ± 0.47	99.33 ± 0.25
101.642 ± 0.87	—	101.47 ± 0.88	99.27 ± 0.323	99.412 ± 0.34	—	99.37 ± 0.31	99.32 ± 0.38
100.92 ± 1.14	—	98.55 ± 0.71	99.1 ± 0.28	99.21 ± 0.17	—	99.3 ± 0.47	99.462 ± 0.21
100.5 ± 0.792	102.09 ± 0.97	98.01 ± 0.66	98.95 ± 0.42	99.03 ± 0.56	101.7 ± 0.85	99.27 ± 0.34	99.26 ± 0.23

[e] Labeled to contain 20 mg ephedrine.HCl, 140 mg theophylline, 90 mg guaiphenesin, 12.5 mg diphenhydramine.HCl per tablet (Misr, Egypt).
[f] Labeled to contain 24 mg phenylpropanolamine.HCl, 400 mg paracetamol, and 3 mg chlorpheniramine maleate per tablet (Kahira Pharmaceuticals, Egypt).
[g] Labeled to contain 250 mg methyldopa per tablet (Merck Sharp and Dohme).

sional stirring for 30 min. About 10 mL 10% potassium iodide solution was added and the liberated iodine was titrated with 0.02N sodium thiosulfate solution, using starch as indicator. A blank experiment carried out under identical conditions omitted the active ingredients.

Procedure B: Pure Drugs, Modified Back-Titration

The oxidation was started in alkaline medium (pH 11.0 phosphate buffer for ephedrine or norephedrine and pH 7.4 with methyldopa). To a known volume of drug, a measured excess of organic brominating solution and 10 mL pH 11.0 or 7.4 phosphate buffer were added and the solution was diluted to 50 mL. After 20 min, 20 mL 2N HCl or H_2SO_4 was added and the unconsumed organic brominating agent was determined iodimetrically. A blank experiment carried out under identical conditions omitted the active ingredients.

Also using pH 11.0 and 7.4 phosphate buffers, ephedrine, norephedrine, and methyldopa were determined potentiometrically using a combination of platinum and calomel electrodes.

Procedure C: Pure Drugs, Spectrophotometric Method

An accurately measured volume of solution equivalent to about 2.0 mg ephedrine.HCl or 1.9 mg norephedrine.HCl in water was transferred to small separating funnel. Then 10 mL phosphate buffer pH 11.0 and 2 mL brominating agent (0.2M) were added, the mixture was shaken for 20 min, and 1-2 drops of concentrated HCl was added. (The acid converts amines which are not oxidized by the brominating agents to their non-extractable salts.) The mixture was shaken for 30 s with 20 mL hexane, and the hexane layer was filtered through Whatman No. 1 paper; the volume was adjusted to 25 mL with hexane in a 25 mL volumetric flask. The absorbance of the hexane extract was determined at 242 nm using hexane as a blank.

For methyldopa, an accurately measured volume of solution equivalent to about 0.65 mg was added to a 10 mL volumetric flask, 0.5-1 mL brominating agent (0.02M) was added, the volume was adjusted with (pH 7.4) phosphate buffer, and the solution was left for 20 min. The absorbance of the solution was measured at 485 nm against a blank prepared in an identical manner but omitting the addition of methyldopa. The concentrations of methyldopa, ephedrine.HCl, and norephedrine.HCl were calculated by reference to calibration graphs prepared in the same manner, using concentrations of 0.04-0.65 mg methyldopa, 0.02-2 mg ephedrine.HCl, and 0.15-1.9 mg norephedrine.HCl.

Procedure D: Dosage Forms

(a) *Tablets.*—Twenty tablets were weighed and powdered, and a portion equivalent to 20 mg active ingredient was accurately weighed, extracted by a minimum of either water or 0.1N sulfuric acid, and mechanically shaken 15 min. The extract was filtered into a 50 mL volumetric flask and diluted to volume with water or 0.1N sulfuric acid. An aliquot of this solution was analyzed using procedures A, B, and C.

(b) *Injections and solutions.*—An aliquot of the liquid preparation containing about 10 mg active ingredient was accurately pipetted into a 50 mL volumetric flask and diluted to volume with water. An aliquot of this solution was analyzed according to procedures A, B, and C.

(c) *Syrups.*—An aliquot of syrup containing about 10 mg active ingredient was accurately pipetted into a separator containing 1 mL 1N sulfuric acid, and extracted with 10 mL chloroform. The extract was discarded and 5 mL potassium carbonate solution (1 + 5) was added. After gas evolution ceased, the solution was extracted with three 10 mL portions of chloroform which were combined in a second separator. The chloroform solution was extracted with 25 mL 0.1N sulfuric acid, and the acid layer was filtered through paper and diluted to 50 mL with 0.1N sulfuric acid (11). An aliquot of the prepared solution was analyzed using procedures A, B, and C.

Discussion

One of the more selective analytical procedures for phenethanolamine drugs was that described by Heimlich and co-workers (12). They determined phenylpropanolamine in urine by oxidizing the drug to benzaldehyde with periodate, and measuring the ether-extracted aldehyde spectrophotometrically. The reaction of *N*-bromosuccinimide with these drugs led to the observation that alkaline solutions of *N*-bromosuccinimide or the hypohalite ion effect facile cleavage of phenethanolamines to benzaldehyde, when the pH is increased to 12 with sodium carbonate; this was confirmed by Chafetz et al. (13). Our study revealed that ephedrine and norephedrine.HCl are successfully oxidized to benzaldehyde not only with *N*-bromosuccinimide but also with some other *N*-haloimides: *N*-bromophthalimide, bromamine-T, and dibromohydantoin in pH 11.0 phosphate buffer.

Table 3. Results for determination of ephedrine and norephedrine at pH 11.0 and methyldopa at pH 7.4 phosphate buffer, using potentiometric and back-titration procedures [a]

Drug	Range, mg	Recovery with NBS, %		Recovery with DBH, %		Recovery with NBP, %		Recovery with BAT, %	
		Potentio.	Back-titrn	Potentio.	Back-titrn	Potentio.	Back-titrn	Potentio.	Back-titrn
Ephedrine.HCl	4.04–60.6	99.19 ± 0.2	98.82 ± 0.242	99.21 ± 0.2	98.932 ± 0.3	98.93 ± 0.11	98.11 ± 1.33	99.16 ± 0.17	98.89 ± 0.25
t [b]		2.81	0.682	4.08	0.234	0.38	1.483	2.74	0.085
F [c]		4.06	4.97	2.7	16.4	4.15	5.338	6.28	1.73
Norephedrine.HCl	3.942–56.13	99.17 ± 0.44	99.00 ± 0.68	99.45 ± 0.4	99.02 ± 0.67	98.8 ± 0.43	98.78 ± 0.55	99.24 ± 0.3	99.03 ± 0.66
t		1.44	0.363	0.59	0.44	0.56	0.53	2.55	0.482
F		6.29	1.57	6.411	3.44	4.17	2.11	2.54	4.06
Methyldopa	4.22–33.76	99.242 ± 0.38	99.36 ± 0.21	99.27 ± 0.38	99.372 ± 0.22	98.56 ± 0.622	99.082 ± 0.34	99.29 ± 0.403	99.44 ± 0.32
t		2.971	5.29	4.6	5.22	1.5	2.18	3.07	4.61
F		5.061	2.18	6.26	3.53	1.165	6.26	2.12	1.2

[a–c] See footnotes, Table 1.

Table 4. Results for determination of ephedrine.HCl and norephedrine.HCl at pH 11.0 and methyldopa at pH 7.4 phosphate buffer by potentiometric and back-titration procedures for pharmaceutical preparations [a]

Sample	Active ingredient	Recovery with NBS, %		Recovery with BDH, %		Recovery with NBP, %		Recovery with BAT, %	
		Potentio.	Back-titrn	Potentio.	Back-titrn	Potentio.	Back-titrn	Potentio.	Back-titrn
Ephedrine tablets [b]	ephedrine.HCl	99.21 ± 0.62	99.472 ± 0.37	99.22 ± 0.62	99.66 ± 0.83	98.98 ± 0.6	99.65 ± 1.34	99.22 ± 0.63	99.88 ± 0.68
Ephedrine ampules [c]	ephedrine.HCl	99.21 ± 0.64	99.85 ± 0.75	99.22 ± 0.67	98.86 ± 0.74	98.89 ± 0.57	100.01 ± 1.08	99.26 ± 0.62	99.45 ± 0.4
Asmacid tablets [d]	ephedrine.HCl	99.364 ± 0.47	99.9 ± 0.73	99.38 ± 0.46	99.93 ± 0.702	99.044 ± 0.66	100.08 ± 1.4	99.4 ± 0.46	99.92 ± 0.7
Tussipect syrup [e]	ephedrine.HCl	99.3 ± 0.39	99.241 ± 0.462	99.33 ± 0.39	99.29 ± 0.44	98.984 ± 0.45	98.9 ± 0.58	99.33 ± 0.39	99.32 ± 0.39
Noflu tablets [f]	phenyl-propanol-amine.HCl	99.24 ± 0.3	99.38 ± 0.57	99.312 ± 0.27	99.4 ± 0.59	98.595 ± 0.8	99.21 ± 0.59	99.3 ± 0.241	99.395 ± 0.58
Aldomet tablets [g]	methyldopa	99.11 ± 0.3	99.1 ± 0.27	99.112 ± 0.27	99.16 ± 0.21	98.36 ± 0.57	98.774 ± 0.262	99.14 ± 0.26	99.13 ± 0.22

[a] Each result is mean of 6 experiments ± 95% confidence interval.
[b–g] Samples as in Table 2.

Table 5. Results for spectrophotometric determination of ephedrine.HCl, norephedrine.HCl at pH 11.0 and methyldopa at pH 7.4 phosphate buffer, using NBS, DBH, NBP and BAT [a]

Drug	Range, mg	Recovery, % NBS	DBH	NBP	BAT
Ephedrine.HCl	0.2–2	99.34 ± 0.38	99.36 ± 0.36	99.17 ± 0.25	99.37 ± 0.34
t [b]		2.67	2.88	1.274	3.133
F [c]		2.35	2.025	3.543	1.732
Norephedrine.HCl	0.15–1.9	99.49 ± 0.33	99.49 ± 0.3	99.242 ± 0.532	99.492 ± 0.29
t		4.1	4.33	1.55	4.49
F		10.69	2.283	0.87	3.503
Methyldopa	0.04–0.65	99.45. ± 0.23	99.46 ± 0.25	99.272 ± 0.29	99.46 ± 0.24
t		5.71	4.48	3.73	5.651
F		1.36	1.57	2.38	1.21

[a–c] See footnotes, Table 1.

Freshly prepared *N*-haloimides provided about 97–100% yield of benzaldehyde with phosphate buffer, which is much better than the yield for sodium carbonate. These active ingredients can be determined titrimetrically in 20% hydrochloric acid medium using the visual indicator methyl orange or methyl red. These indicators give a sharp change at the equivalence point. Potentiometric as well as back-titration procedures are applied for the determination of these active ingredients in pure form and in pharmaceutical preparations; the results are shown in Tables 1 and 2. The reaction proceeds faster in pH 11.0 phosphate buffer than in acid medium, as applied to the determination of ephedrine and norephedrine.HCl potentiometric and back-titration methods; the results are shown in Tables 3 and 4 for pure drugs and dosage forms.

Addition of brominating agents to a solution of ephedrine or norephedrine.HCl at pH 11.0 phosphate buffer causes cleavage to form benzaldehyde; benzaldehyde can then be extracted with hexane and will absorb at 242 nm. The reaction can be expressed by the following equation:

$$C_6H_5\text{-}CHOH\text{-}CH(NH)_2\text{-}CH_3 + 2\ BrO^- + OH^- \rightarrow C_6H_5\text{-}CHO + CH_3\text{-}CO_2^- + NH_3 + 2\ Br^- + H_2O$$

The benzaldehyde produced can also be identified by using thin layer chromatography on silica gel and chloroform as solvent. It is characterized by its UV fluorescence and 0.76 R_f value (14).

Effect of Catecholamines

Epinephrine, norepinephrine, and isoproterenol are 3,4-dihydroxyphenethanolamine derivatives which might be expected to undergo oxidation to 3,4-dihydroxybenzaldehyde. These compounds, however, are preferentially oxidized by cyclization to adrenochrome. Under the Abou Ouf et al. (6) conditions, the compounds are immediately oxidized in the aqueous phase to red compounds which are not extractable with hexane.

These results were confirmed here by the oxidation of methyldopa to the corresponding aminochrome. The equivalence point in the ti-

Table 6. Results for spectrophotometric determination of ephedrine.HCl, norephedrine.HCl, and methyldopa in pharmaceutical preparations [a]

Samples	Active ingredient	Recovery, % NBS	DBH	NBP	BAT
Ephedrine tablets [b]	Ephedrine.HCl	99.39 ± 0.263	99.394 ± 0.26	98.974 ± 0.302	99.4 ± 0.25
Ephedrine ampules [c]	Ephedrine.HCl	99.332 ± 0.32	99.344 ± 0.31	98.982 ± 0.38	99.35 ± 0.31
Asmacid tablets [d]	Ephedrine.HCl	99.382 ± 0.282	99.39 ± 0.28	99.26 ± 0.37	99.39 ± 0.02
Tussipect syrup [e]	Ephedrine.HCl	99.35 ± 0.4	99.7 ± 0.4	98.97 ± 0.65	99.354 ± 0.4
Noflu tablets [f]	Phenylpropanolamine.HCl	99.12 ± 0.52	99.13 ± 0.52	99.912 ± 0.532	99.13 ± 0.52
Aldomet tablets [g]	Methyldopa	99.31 ± 0.23	99.27 ± 0.23	99.074 ± 0.271	99.32 ± 0.22

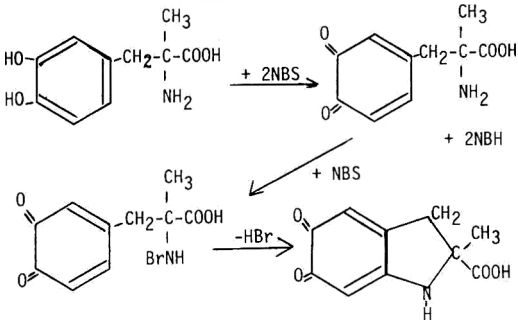
[a] Each result is mean of 6 experiments ± 95% confidence interval.
[b–g] Samples as in Table 2.

tration corresponds to the addition of 3 moles of BAT, NBS, NBP or 1.5 mole of DBH per mole of methyldopa. It appears from the results that a simple procedure could be devised in which the self-indicating property of methyldopa might be used for its determination. The titrations are also carried out using visual indicators (methyl orange or methyl red). These indicators give a very sharp change at the equivalence point. A potentiometric method can also be used; typical results are shown in Tables 1 and 2.

The absorption spectrum of methyldopa in 0.01N HCl shows a maximum at 280 nm. The addition of the brominating agent to a buffered solution of methyldopa produces a new spectrum corresponding to aminochrome, with an absorption maximum at 485 nm. Spectrophotometric results are shown in Tables 5 and 6.

Thin layer chromatography is used to identify aminochrome on silica gel in a butanol–acetic acid–water (66 + 17 + 17) solvent system (14); the R_f value is 0.55.

The study of the stoichiometry of the reactions revealed that the reaction between methyldopa and brominating agents can be represented by the following scheme:



Conclusion

We have shown that ephedrine.HCl, norephedrine.HCl, and methyldopa can be determined spectrophotometrically and titrimetrically using organic brominating agents.

The results in Tables 1, 3, and 5 were subjected to a paired comparison test (15) in which calculated values for Student's *t*-test were compared for the proposed and official methods. Student's *t*-test shows no significant differences. Each of the methods was applied successfully to the determination of these active ingredients as raw materials and in their dosage forms.

References

(1) Higuchi, T., & Bodin, J. I. (1961) in *Pharmaceutical Analysis*, T. Higushi & E. Brochmann-Hanssen (Eds), Interscience Publishers, New York, NY, p. 313
(2) Burke, D., Venturell, V. S., & Senkowski, X. (1974) *J. Pharm. Sci.* **63**(2), 269
(3) Masatosh, Y., Yoshito, Y., Kazuya, K., & Yosuke, O. (1979) *Anal. Chim. Acta* **108**, 297–308
(4) Supplement (1975) to the *Pharmacopoeia of India*, The Controller of Publications, Delhi, p. 139
(5) Ramana Rao, G., & Sivarama Krishnan, M. V. (1978) *India J. Pharm.* **40**(1), 18
(6) Abou Ouf, A., Walash, M. I., & Salem, F. B. (1981) *Analyst* **106**, 949–954
(7) Nair, C. G. R., Lalithakumari, R., & Senan, P. I. (1954) *Talanta* **55**, 525
(8) Barakat, M. Z., & Abdel-Wahab, M. F. (1973) *Anal. Chem.* **26**, 1954
(9) Saadia, M. M., (1980) Master's Thesis, Mansoura University, Mansoura, India
(10) Burry, S., Kwit, D., & Jawarska, R. (1966) Polish Pat. 51-580; *Chem. Abstr.* (1967) **67**, 90811a
(11) *U.S. Pharmacopeia* XX (1980) U.S. Pharmacopeial Convention, Rockville, MD, pp. 276, 517, 619
(12) Heimlich, K. R., MacDonnell, D. R., Flanggan, T. L., & O'Brien, P. P. (1961) *J. Pharm. Sci.* **50**, 232
(13) Chafetz, L., Grosser, L. A., Schriftman, H. & Daly, R. (1970) *Anal. Chim. Acta* **52**(2), 370
(14) Desimio, M. (1962) *Boll. Soc. Ital. Farm. Osp.* **8**, 155; cited in *Pharm. Acta Helv.* (1963) **38**, 383, through *Thin-Layer Chromatography*, 2nd Ed. (1969) E. Stahl (Ed.), p. 499
(15) Hinchen, J. D. (1969) *Practical Statistics for Chemical Research*, Methuen, London, UK, p. 22

PESTICIDE FORMULATIONS

Gas-Liquid Chromatographic Derivatization and Chromatography of *N*-Methylcarbamate Methoxy Derivatives Formed with Trimethylanilinium Hydroxide

LEONARD OGIERMAN
Institute of Plant Protection, 44153 Sosnicowice, Poland

Carbamate insecticides injected into a gas-liquid chromatograph react with trimethylanilinium hydroxide to give derivatives that have good gas-liquid chromatographic properties. The substituted phenyl *N*-methylcarbamates give methoxy derivatives by this procedure. Chromatographic response was linear with increased concentration for the synthetic standard and the on-column methylation products of the examined substances. The standards obtained were identified by ultraviolet and infrared absorption spectroscopy, as well as by nuclear magnetic resonance and mass spectral analysis.

Because of their insecticidal activity, the *N*-methylcarbamates are commonly used in plant protection. Many chromatographic methods have been developed for determining the active substances in formulations. However, carbamate insecticides present some difficult analytical problems because of their instability and low volatility in gas-liquid chromatographic (GLC) columns. This group of pesticides has been analyzed by GLC after their stable methyl- (1–3) or perfluoro-derivatives were prepared (4, 5).

Derivatization by the flash-heater reaction with trimethylanilinium hydroxide provides rapid qualitative and quantitative GLC of the *N*-methyl- and *N*-arylcarbamates (5), and also the *N*-methyl oxime carbamates (1). This paper reports the applicability of this methylation technique to the GLC analysis of a series of the substituted phenyl *N*-methylcarbamate insecticides.

Experimental

Materials

Pesticide standards were obtained from commercial sources and were further purified by recrystallization from an *n*-hexane-benzene system. Carbamates investigated were carbofuran, methiocarb, and propoxur (Bayer AG, Levenkusen, GFR); meobal (Sumitomo, Osaka, Japan); tsumacide (Nihon Nohyaku, Tokyo, Japan); dioxacarb (Ciba-Geigy SA, Basel, Switzerland); and bendiocarb (Fisons Ltd, Hauxton, UK). Trimethylanilinium hydroxide (TMAH) was purchased as a 0.5M methanolic solution (Pierce Chemical Co., Rockford, IL 61105).

Preparation of Methoxy Derivatives

The anisole derivatives were obtained in a conventional way from the corresponding carbamates: Carbamates (1.0 g) were dissolved in 100 mL 0.1M methanolic NaOH solution. The mixture was heated to 60°C and stirred ca 0.5 h at that temperature to ensure complete hydrolysis of carbamates. Then, the solution was cooled to about 30°C. Methyl iodide (4.5 g) was dissolved in 50 mL methanol and then added with stirring to the hydrolysis mixture. The flask was heated to 45°C, the reaction mixture was filtered hot, and the residue was dissolved in 200 mL chloroform. The chloroform solution was washed 3 times with 200 mL water and dried with anhydrous sodium sulfate. Chloroform was removed by evaporation under a stream of dry air.

The methoxy derivatives were additionally purified by preparative thin layer chromatography (TLC). Chromatograms were developed by using an *n*-hexane–benzene–acetone (3 + 1 + 1) solvent mixture on silica gel 60-F_{254} plates (E. Merck, Darmstadt, GFR).

Apparatus

Several types of instruments were used to identify the derivatives. Ultraviolet (UV) and infrared (IR) spectra were obtained with Pye Unicam SP-1800 and SP-200 spectrophotometers, respectively. Nuclear magnetic resonance (NMR) spectra were measured at 60 Hz with a Jeol spectrometer, and mass spectra (MS) were recorded with an LKB-9000s mass spectrometer.

Optimization of Reaction with TMAH

The carbamates under investigation were prepared as 0.05M methanolic solutions. One

Received June 15, 1981. Accepted April 1, 1982.

0004-5756/82/6506-1452-05$01.00

mL aliquots of these solutions were added to 10 mL volumetric flasks and TMAH solution was added to prepare 1:1 to 5:1 dilutions of TMAH-carbamate. Each solution was diluted to volume with methanol. Thus, the solutions prepared for optimization of the reaction contained TMAH as well as 0.05 mmole of a given carbamate in 10 mL methanol. For each determination, a 4 μL (0.02 μmole) aliquot of the TMAH-carbamate solution was injected into the gas chromatograph. The quantity of TMAH giving the maximum carbamate reaction was estimated by using a standard curve that was prepared from the authentic samples of the methoxy anisole derivatives.

Linearity of Response and Calibration Graphs

Standard methanolic solutions of the methoxy derivatives of the corresponding carbamates, ranging in concentration from 5 to 60 ng/μL, were prepared for determination of linearity of response. Response with regard to the flash-heater reaction was then estimated by using methanolic solutions of 10–60 ng carbamates/μL (an approximate molar ratio of 5:1 of TMAH-pesticides).

Gas-Liquid Chromatography

A Perkin-Elmer 900 chromatograph with a flame ionization detector (FID) was used for each analysis. The glass column (6 ft × 3 mm id) was packed with 3% EGSP-Z on 80–100 mesh Gas-Chrom Q and conditioned at 220°C; detector and injector temperatures were 220 and 260°C, respectively. Flow rates of argon, hydrogen, and air were 40, 35, and 180 mL/min, respectively. The isothermal analysis was performed at 160°C.

Preparation of Solutions and Sample

Applicability of the proposed derivatization method for analysis of the *N*-methylcarbamate pesticides was studied by using carbofuran (Furadan®).

Standard solution.—Weigh 100 mg methoxy carbofuran derivative into 100 mL volumetric flask, dilute to volume with 50 mL internal standard solution and 50 mL methanol, and mix.

Internal standard solution.—Dissolve 1 g propiophenone (Fluka AG, Buchs, Switzerland) in 1 L methanol.

Extraction of carbamate.—Accurately weigh portion of well mixed sample containing ca 100 mg carbofuran formulation into 150 mL glass-stopper Erlenmeyer flask. Pipet 100 mL internal standard solution into flask and agitate 0.5 h on magnetic stirrer to dissolve active ingredient. Filter extract through medium paper and use filtrate for GLC analysis. Use syringe to add 5 mL filter extract and 5 mL 0.05M TMAH into ampule with silicone stopper. This sample contains 50 mg internal standard and a molar excess of TMAH per 100 mL solution.

Determination

Make 4 replicate alternate injections of 4 μL sample and 4 μL standard solution into gas chromatograph operated under conditions described above. Calculate peak area ratios for samples and standards, and determine carbofuran content as follows:

$$\% \text{ Carbofuran} = (A/A') \times (W'/0.805\ W) \times P$$

where A and A' = average peak area ratios of carbofuran to propiophenone for sample and standard, respectively; W and W' = mg sample and standard, respectively; 0.805 = molar ratio of methoxy derivative to carbofuran, and P = % purity of standard. Equation is correct for quantitative derivatization of carbofuran to methoxy derivative.

Results and Discussion

Carbamate pesticides present inherent difficulties in GLC analysis. Under gas chromatographic conditions, carbamates are partially transformed to the corresponding phenols, which makes quantitative interpretation of results fairly difficult. In the present study we solved this problem by quantitative transformation of carbamates to phenols with the simultaneous methylation of these products to the respective methoxy derivatives. Phenyl *N*-methylcarbamates give methoxy derivatives (anisoles) by this procedure

$$\text{Ar-OCONHCH}_3 \xrightarrow{\text{TMAH}} \text{Ar-OCH}_3$$

With the experimentally established molar ratio of TMAH to carbamate, the reaction is almost quantitative, and is highly reproducible. The molar ratio of TMAH–pesticide of 2:1 provides the maximum transformation of these substances to their derivatives. The results in this paper were obtained for the molar ratio of 5:1. In the studied temperature range of the injection chamber, i.e., from 220 to 260°C, there was no difference in carbamate methylation yields. Further lowering of temperature below 220°C was limited by the applied detector temperature. Transformation yields were 98.5% (bendiocarb, carbofuran, dioxacarb, and pro-

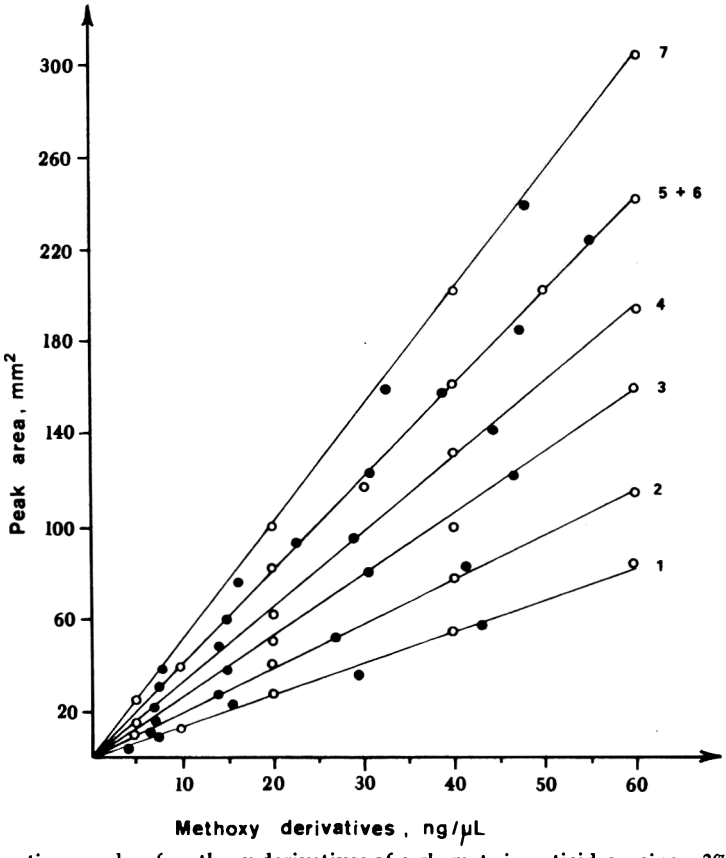


Figure 1. Calibration graphs of methoxy derivatives of carbamate insecticides, using a 3% EGSP-Z column. Methoxy derivatives of (1) tsumacide, (2) meobal, (3) propoxur, (4) carbofuran, (5) bendiocarb, (6) dioxacarb, (7) methiocarb. ○, Standard methoxy derivatives; ●, TMAH-carbamate (5:1).

poxur), and approximately 99% (meobal, methiocarb, and tsumacide).

Figure 1 shows the detector response curves (calibration graphs) of the anisole derivatives prepared from the standards. The flash-heater reaction method was tested to determine whether a linear response could also be obtained with increased concentration of the anisole derivative. Chromatographic response for the compounds was linear with increased concen-

Table 1. Chemical names and R_f values for carbamates and their methoxy derivatives

Common name	Chemical name	R_f value	
		Carbamate	Anisole
Dioxacarb	2-(1,3-Dioxolan-2-yl)phenyl-*N*-methylcarbamate	0.12	
	2-(1,3-Dioxolan-2-yl)anisole		0.47
Bendiocarb	2,2-Dimethyl-1,3-benzodioxol-4-yl-*N*-methylcarbamate	0.21	
	2,2-Dimethyl-4-methoxy-1,3-benzodioxol		0.65
Carbofuran	2,3-Dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate	0.23	
	2,2-Dimethyl-2,3-dihydro-7-methoxybenzofuran		0.60
Propoxur	2-Isopropoxyphenyl *N*-methylcarbamate	0.26	
	2-Isopropoxyanisole		0.63
Tsumacide	3-Methylphenyl-*N*-methylcarbamate	0.28	
	3-Methylanisole		0.75
Meobal	3,4-Dimethylphenyl-*N*-methylcarbamate	0.31	
	3,4-Dimethylanisole		0.80
Methiocarb	3,5-Dimethyl-4-(methylthio)phenyl *N*-methylcarbamate	0.34	
	3,5-Dimethyl-4-thiomethylanisole		0.77

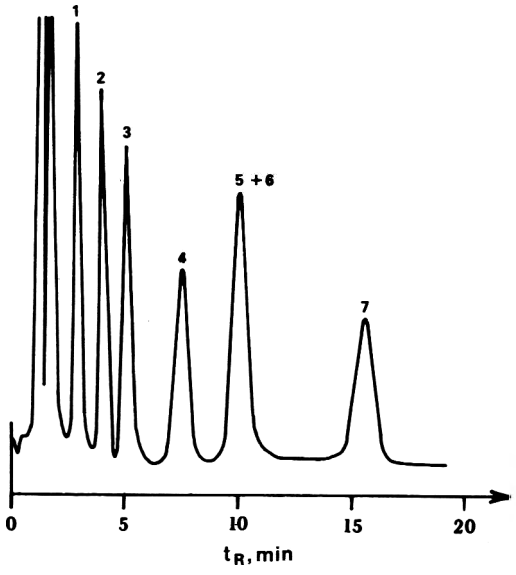


Figure 2. Chromatographic separation of carbamate mixture by on-column derivatization with TMAH. Column temperature 160°C. (1) tsumacide, (2) meobal, (3) propoxur, (4) carbofuran, (5) bendiocarb, (6) dioxacarb, (7) methiocarb.

trations of the methoxy derivatives. The linear behavior of detector (FID) response for the nanogram levels of carbamate with TMAH in on-column methylation allows application of this method to GLC determination of residues of these pesticides in biological material.

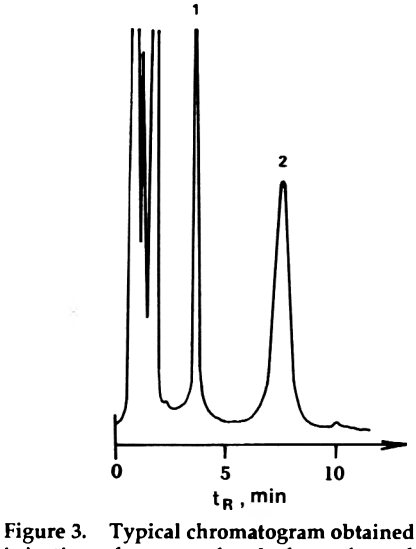


Figure 3. Typical chromatogram obtained from 4 μL injection of extract of carbofuran formulation (Furadan 50 WP): (1) propiophenone (internal standard); (2) carbofuran methoxy derivative.

Table 2. Determination of carbofuran (%) in 3 commercial formulations [a]

	Concn (%) of active substance		
Detn	25	50	75
1	25.16	51.25	76.16
2	25.08	50.82	76.35
3	25.42	51.16	76.28
4	25.40	51.40	76.50
5	25.21	50.58	76.20
Av.	25.25	51.04	76.29
CV, %	0.735	0.670	0.179

[a] The 98.5% derivatization of carbofuran to the corresponding methoxy derivative was taken into account.

Table 1 gives the R_f values of the carbamates and their derivatives together with their chemical names. The compounds were located on the TLC plates by quenching of the fluorescent indicator in the silica gel at 254 nm.

Chromatographic separation of a mixture of the flash-heater methylation products of the *N*-methylcarbamates is given in Figure 2. Application of the polar stationary phase EGSP-Z (McReynolds constant $\Sigma\Delta I = 2278$) enables isothermal separation of the carbamate pesticides.

The applicability of TMAH on-column methylation of the pesticides to the analysis of formulations was demonstrated by determining carbofuran in formulations. Results (Figure 3 and Table 2) were satisfactory.

Table 3. Characteristics of UV, IR, and NMR spectra of the carbamate methoxy derivatives

Methoxy derivative of phenyl *N*-methylcarbamate	UV (nm) λ max.	IR (cm^{-1}) ν OCH_3	NMR (ppm) 3H, singlet
Bendiocarb	222	1260	3.82
	274	1045	
	278		
Carbofuran	226	1280	3.78
	278	1075	
	286		
Dioxacarb	218	1255	3.84
	256	1030	
	274		
	282		
Meobal	224	1275	3.68
	278	1080	
	286		
Methiocarb	236	1275	3.70
	264	1050	
	286		
Propoxur	226	1230	3.75
	278	1075	
Tsumacide	222	1250	3.72
	274	1040	
	282		

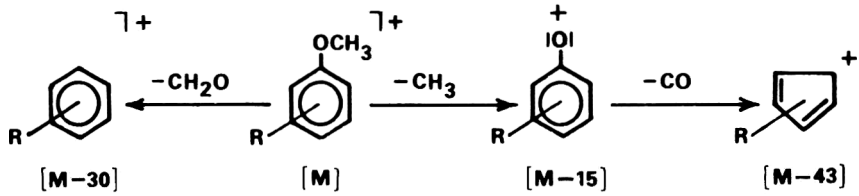


Figure 4. Basic MS fragmentation pattern for methoxy derivatives.

Identification of Methoxy Derivatives

The obtained standards were spectroscopically identified in the UV and IR ranges, and also by means of NMR and MS analyses. Table 3 shows absorption maxima of the methoxy derivatives analyzed as methanol solutions (10 ppm) in the UV range. Interpretation of the IR spectra (CCl_4 solutions) was limited to the wavelength ranges in which absorption caused by valency vibrations of the methoxy (—OCH_3) group was observed. Data for NMR spectra of the examined derivatives dissolved in CCl_4 are also shown in Table 3 as the resonance signal ranges of the 3H protons belonging to the methoxy group.

The basic MS fragmentation patterns of the methoxy derivatives are shown in Figure 4. With the methoxy derivatives that include heterocyclic systems (e.g., bendiocarb, carbofuran, and dioxacarb), fragmentation is much more complex. The parent peaks of the methoxy derivatives correspond to the respective weights. The intensity of the fragmentation peaks $[M - 15]^+$, which appears as 20 to 100% of the main peak, is characteristic of the phenolate ions and confirms the chemical structure of the obtained derivatives, as well as the fragmentation pattern shown.

The results concerning identification of the methoxy carbofuran and propoxur derivatives are a continuation of the previous performed work (3).

Conclusion

We have applied flash-heater derivatization with trimethylanilinium hydroxide of selected *N*-methylcarbamate insecticides to qualitative identification and quantitative GLC determination of the methoxy derivatives. The selectivity of this reaction is preserved by using the molar ratio of TMAH to carbamate equal to 5:1, with the injection chamber temperature at 260°C. The proposed derivatization method was used to analyze carbofuran in formulations. The linear behavior of detector response at nanogram levels allows use of this method for determining residues of these pesticides by gas-liquid chromatography. Application of the method to residue analysis will be reported in a later paper.

References

(1) Bromilow, R. H., & Lord, K. A. (1976) *J. Chromatogr.* **125,** 495–502
(2) Cochrane, W. P. (1979) *J. Chromatogr. Sci.* **17,** 124–134
(3) Wien, R. G., & Tanaka, F. S. (1977) *J. Chromatogr.* **130,** 55–63
(4) Lawrence, J. F. (1976) *J. Chromatogr.* **123,** 287–294
(5) Ryan, J. J., & Lawrence, J. F. (1977) *J. Chromatogr.* **135,** 117–122

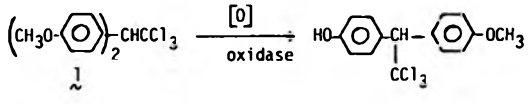
High Performance Liquid Chromatographic Analysis of Impurities and Degradation Products of Methoxychlor

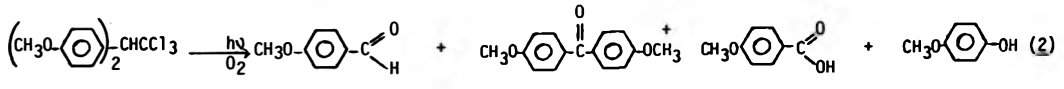
PAUL R. WEST, SUNIL K. CHAUDHARY, GRAHAM R. BRANTON, and REGINALD H. MITCHELL

University of Victoria, Department of Chemistry, Environmental Toxicology Group, PO Box 1700, Victoria, British Columbia, Canada V8W 2Y2

Both reverse and normal phase high performance liquid chromatographic techniques have been applied to the analysis of technical methoxychlor. Facile detection of high molecular weight components yields information that is complementary to the gas chromatographic analysis of the technical pesticide and environmentally altered samples.

Methoxychlor, 1,1,1-trichloro-2,2-bis[*p*-methoxyphenyl]ethane, **1**, a moderately biodegradable and less acutely toxic analog of DDT has been widely adopted in both domestic applications (1), and in control of black fly larvae and elm bark beetle infestations (2). Consequently, there has been renewed interest in both the composition of technical formulations (3–5) and also metabolic (6) and environmental (abiotic) degradation of methoxychlor (7). From an analytical viewpoint, these latter processes tend to increase the polarity and the molecular weight of components in the sample. Specifically, demethylation at the methoxyl group generates the polar phenol, shown in Equation *1* (6). Photooxidation leads to a slate of polar products, Equation *2* (7).





Recent elegant work on the gas chromatography–mass spectrometry of the commercial pesticide (3) has revealed nearly 50 components in the complex technical product. Among these were several high molecular weight components including nonchlorinated tetra-anisylethylene, $(An)_2C{=}C(An)_2$, MW 452.

We now report the complementary advantages of reverse and normal phase liquid chromatography in determination of polar and high molecular weight constituents in methoxychlor formulations and environmentally degraded pesticide.

Experimental

Instruments

High pressure liquid chromatography (HPLC).—A Varian Model 5000 HPLC system equipped with Rheodyne 7125 syringe loading injector (10 and 200 μL loops) was used. For analytical studies, Varian MCH-10 (4 mm × 30 cm) columns were used in reverse phase operation and Si-10 (4 mm × 30 cm) in normal phase work. The detector used was the Varichrom variable wavelength UV detector, normally operated at 254 nm where response for methoxychlor derivatives was optimum. Data processing used the Varian CDS-111 system.

Gas chromatography (GC).—A Varian 3700 gas chromatograph equipped with a flame ionization detector was used. Columns were 30 m × 0.25 mm id SE-54 and SP 2250 open-tubular glass capillary columns supplied by Supelco Inc. (Bellefonte, PA) and 30 mm × 0.20 mm id SP 2100 fused silica WCOT columns supplied by Hewlett-Packard, Inc. For all analyses, the injection port temperature was maintained at 260°C and the column was cooled to ambient temperature (27°C). At 1 min past injection, the temperature was increased to 60°C and the injection port was back-flushed. The column temperature was then programmed at either 5 or 10°C/min to the maximum for the column.

Gas chromatography–mass spectroscopy.—Electron impact spectra were recorded on a Finnigan 3300 GC/MS system equipped with the INCOS data system. Spectra reproduced in this paper represent direct printout of ion chromatogram data. The GC conditions were as follows: col-

Received February 11, 1982. Accepted April 1, 1982.

0004-5756/82/6506-1457–14$01.00

umn 30 m × 0.20 mm id SP 2100 supplied by Hewlett-Packard, Inc.; standard Grob-type injection with oven temperature increased to an initial temperature of 80°C 2 min after injection; after 6 min, the temperature was programmed at a rate of 6°/min to completion at 260°C. Spectra were recorded at both 25 eV and 70 eV ionization energies (nominal values).

GC/MS–chemical ionization.—In initial structure determination studies, chemical ionization (CI) mass spectra were recorded on a Finnigan 3300 GC/MS system equipped with a methane CI source. As above, samples were injected under standard Grob-type conditions, and the column was programmed as above except at 10°C/min.

Nuclear magnetic resonance spectroscopy (^1H-NMR).—NMR spectra were recorded in $CDCl_3$ (unless otherwise stated) on a Perkin-Elmer R-12A (60 MHz) or R32-B (90 MHz) spectrometer, and are reported in ppm downfield from TMS as internal standard. High field spectra used in assignment of high molecular weight impurities and pesticide conjugates were recorded on a WP 250 Bruker 250 MHz spectrometer.

Materials

HPLC.—Solvents were HPLC grade, distilled in glass (Burdick & Jackson Laboratories, Inc.), used as received: *n*-hexane, methylene chloride, methanol, acetonitrile. With the Varian 5000 system, solvent degassing was unnecessary in routine analysis. For reverse phase, water was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ).

Methoxychlor.—Laboratory grade material was purchased from Sigma Chemical Co. Two locally available garden formulations used were Later's 10% Methoxychlor Dust (Later's Chemical Co. Ltd, Richmond, BC, Canada) and Greenleaf Methoxychlor 25% E.C. Domestic (Greenleaf Garden Supplies, Burnaby, BC, Canada).

Methoxychlor impurity sample.—Recrystallization of 100 g technical methoxychlor (nominally 90% *p,p'*-DMDT; Sigma) from chloroform–ethanol (40 + 100) yielded 76 g of the bis-*p*-isomer, 99.8% pure as determined by normal phase HPLC and by gas chromatography (Varian 3700, above). The 24 g residue was hence deemed to have negligible loss of the original components and is referred to as "methoxychlor impurity" in the paper. To inhibit photo-oxidation and photoclosure, the sample was stored at 0°C and shielded from light.

For analysis (reverse phase), 22.8 mg impurity was dissolved in 5 mL HPLC grade methanol, and an 0.8 mL aliquot was combined with 0.2 mL of an internal standard 2,6-dimethoxybenzophenone routinely used to monitor retention times. Assignments are based on retention time of authentic material and also on addition of authentic material to the impurity mixture for confirmation in both GC and HPLC determination. Semiquantitative estimation (HPLC) was based on calibration of the response at 254 nm for a series of standard solutions bracketing the concentration of the pesticide component. Based on duplicate measurements and linearity of the calibration, we estimate < ±10% error in concentrations for the samples used.

Semipreparative Operation

Both normal and reverse phase columns, 8 mm × 50 cm, were used to isolate 1–2 mg quantities of pesticide components. Solutions in CH_3OH (reverse) or CH_2Cl_2 (normal phase) were maintained at 20 mg/200 μL to achieve resolution at the retention time differences (≥0.5 min) encountered in the methoxychlor impurity mixture.

Synthesis of Standards

Standard components used in analytical procedures were synthesized by literature methods cited in Table 1. Commercially available materials including *p*-methoxybenzoic acid, *p*-methoxyphenol, *p,p'*-dimethoxybenzophenone, and *p*-methoxybenzaldehyde were Aldrich reagent grade used as received. Additional compounds were synthesized as follows:

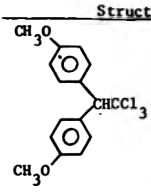
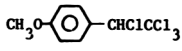
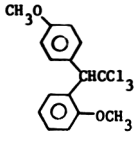
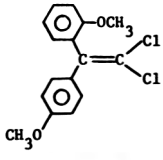
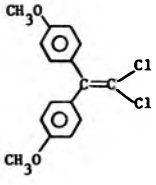
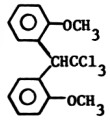
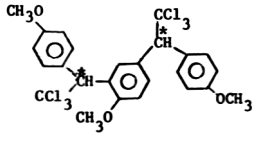
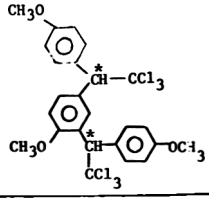
Di(p-methoxyphenyl)ethyne

A solution of phenyllithium (30 mmol) in ca 20 mL ether was added to DMDE, 6 (6.16 g, 20 mmol), in dry ether (250 mL) at ca 20°C under nitrogen (8). The reaction was heated under reflux for 2 h and allowed to stand 12 h before addition of water. The organic layer was washed, dried, and evaporated; the crude product was recrystallized from ethanol and gave 3.5 g pure acetylene (74%), mp 140–142°C (lit. mp 142°C) (9);[1] Hmr (60 MHz) 7.41 (AB, J = 9 Hz, 4H, 2,6-ArH), 6.83 (AB, J = 9 Hz, 4H, 3,5-ArH), and 3.78 (S, 6H, —OCH_3); $M^{+\cdot}$ at m/e 238.

(E)-1,2-Dichloro-1,2-di(p-methoxyphenyl)-ethene, 19

A solution of Cl_2 (1.05 g, 15 mmol) in 25 mL CCl_4 was added dropwise to a solution of di(*p*-methoxyphenyl)ethyne (2.38 g, 10 mmol) in 50 mL CCl_4. After stirring for 3 h at ca 20°C, the solvent was evaporated and the product was chromatographed over silica gel, using cyclo-

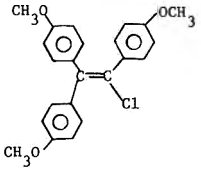
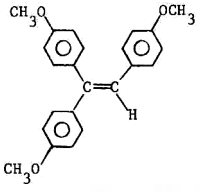
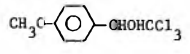
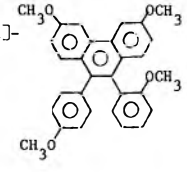
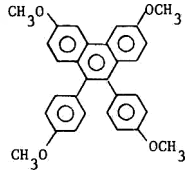
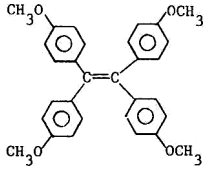
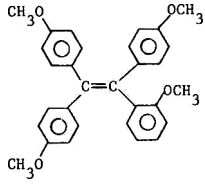
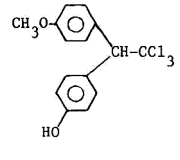
Table 1. Structures of technical methoxychlor constituents detected by normal phase HPLC[a]

	Compound	Structure	Mass Spectrum $M^{+\cdot}$	Ref.
1	Methoxychlor (*p,p'*-DMDT) 1,1,1-trichloro-2,2-bis[*p*-methoxyphenyl]ethane		344	3,4
2	1,1,1,2-Tetrachloro-2-(*p*-methoxyphenyl) ethane		272	3
3	Methoxychlor Isomer (*o,p'*-DMDT)		344	3,4
4	Methoxychlor Olefin Isomer (*o,p'*-DMDE)		308	3,4
5	Unidentified		358[b]	--
6	Methoxychlor Olefin (DMDE)		308	3,4
7	Methoxychlor Isomer (*o,o'*-DMDT) 1,1,1-Trichloro-2,2-bis(*o*-methoxyphenyl) ethane		344	3
8	Condensation Product of *p,p'*-DMDT, 1		580	this work
9	Condensation Product of *o,p'*-DMDT, 2		580	this work

[a] Numbering corresponds to Figure 1.

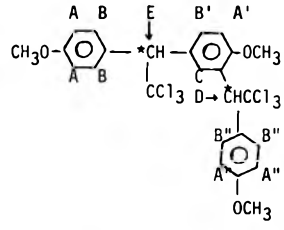
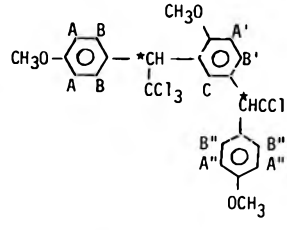
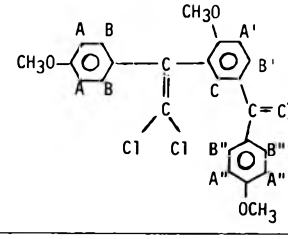
[b] Based on mass spectral analysis of fractions isolated by semi-preparative HPLC.

Table 1. (cont'd)

	Compound	Structure	Mass Spectrum $M^{+\cdot}$	Ref.
10	Unresolved (Two components)			--
11	Chlorotrianisene, TACE 1-chloro-1,2,2-tris[*p*-methoxyphenyl]ethene		380	4,5,9
12	1,2,2-Tris[*p*-methoxyphenyl]ethene		346	this work
13	2,2,2-trichloro-1-(*p*-methoxyphenyl)ethanol		254	3
14	3,6-dimethoxy-9-[*o*-methoxyphenyl]-10-[*p*-methoxyphenyl]-phenanthrene		450	4
15	3,6-dimethyl-9,10-bis(*p*-methoxyphenyl)phenanthrene		450	4
16	1,1,2,2-tetrakis(*p*-methoxyphenyl)ethene (TAE)		452	4
17	1-(*o*-methoxyphenyl)-1,2,2-tris(*p*-methoxyphenyl)ethene		452	4
18	1,1,1-trichloro-2-(*p*-hydroxyphenyl)-2-(*p*-methoxyphenyl)-ethane		330	3[c]

[c] Isolated by semi-preparative HPLC, NMR and MS data identical to reference 3.

Table 2. ^{1}Hmr of high molecular weight methoxychlor condensation products

	Structure[a]	Proton assignment		Chemical shift
8		2B, 2B", B' (mult)	5 H	7.6-7.3
		C (singlet)	1 H	7.25
		2A", 2A, A' (mult)	5 H	6.9-6.7
		D, E (singlets)	2 H	5.67, 5.00
		-OCH_3(3) (singlets)	9 H	3.80, 3.77, 3.75
9		2B, 2B", B' (mult)	5 H	7.6-73
		C (singlet)	1 H	7.25
		2A", 2A, A' (mult)	5 H	6.9-6.7
		D, E (singlets)	2 H	5.67, 4.99
		-OCH_3(3) (singlets)	9 H	3.79, 3.77, 3.74
25		2B, 2B", B', C (mult)	6 H	7.3-7.1
		2A, 2A", A' (mult)	5 H	6.8-6.9
		-OCH_3(3) (singlets)	9 H	3.81, 3.79, 3.73

[a]Compounds 8 and 9 are diastereomers (threo and erythro). Absolute assignments have not been made.

hexane–ethyl acetate (97 + 3) as eluant to give product **19.** Recrystallization from ethanol yielded 1.3 g white crystals (42%), mp 162–166°C (lit. (12) mp 163–164°C, lit. (4) mp 170°C); ^{1}Hmr (60 MHz) 7.42 (AB, J = 9 Hz, 4H, 3,6-ArH), 6.70 (AB, J = 9 Hz, 4H 3,5-ArH), and 3.77 (S, 6H, —OCH_3); UV = λ_{max} (log ϵ max) 283 nm (4.26); $M^{+\cdot}$ at m/e 308.

The residual mother liquors are enriched in the (Z)-isomer, $M^{+\cdot}$ 308, whose retention time corresponds exactly with material produced by the acid-catalyzed isomerization of the pure (E)-isomer at 120°C (13). However, the (Z)-isomer (**20**) could not be induced to crystallize directly from the mother liquors.

(Z)-1,2-Dichloro-1-(o-methoxyphenyl)-2-(p-methoxyphenyl)ethene, 21

NOTE: The (E)- and (Z)-dichlorostilbenes, **21** and **22,** cannot be synthesized by addition to the analogous diarylacetylene because cyclization to a benzofuran occurs during chlorination. Alternatively, the following $SnCl_4$-catalyzed isomerization of *o,p'*-DMDT, **3,** has been developed.

A mixture of *o,p'*-DMDT, **3** (8.5 g, 0.025 mol), and stannic chloride (26.05 g, 0.1 mol) in 150 mL dry benzene was refluxed 10 h. The reaction was then worked up by adding benzene, and the organic layer was washed (water, aqueous $NaHCO_3$, water), dried, and evaporated. The product was chromatographed over neutral alumina (activity II–III) using pentane–benzene (8 + 2) as eluant. Eluted first was 2-chloro-3-(*p*-methoxyphenyl)benzofuran, 0.6 g (10%), as a colorless oil. Eluted next was a mixture of the MW 308 isomers, *o,p'*-DMDE, **4,** and the (E)- and (Z)-stilbenes, **21** and **22,** 3.0 g (40%). The material was subsequently chromatographed over silica gel on a Waters Prep LC500 system using ethyl acetate–cyclohexane (3 + 97) as eluant and pre-conditioner, to yield separated (Z)-stilbene, **21.** Recrystallization from methanol gave white needles (mp 82–83°C), ^{1}Hmr 60 MHz (C_6D_6) 7.3–6.2 (m 9H, ArH) and 3.19 and 3.08 (S, 3H each —OCH_3, unresolved in $CDCl_3$ as a 6H singlet at δ3.70), M^+ at m/e 308 methane CI. Analytically calculated for $C_{14}H_{14}O_2Cl_2$: C, 62.15; H, 4.56; found: C, 62.51; H, 4.67.

Isolation of the (E)-isomer free of the *o,p'*-DMDE under the preparative column conditions was not possible. On heating **21** at 120°C in

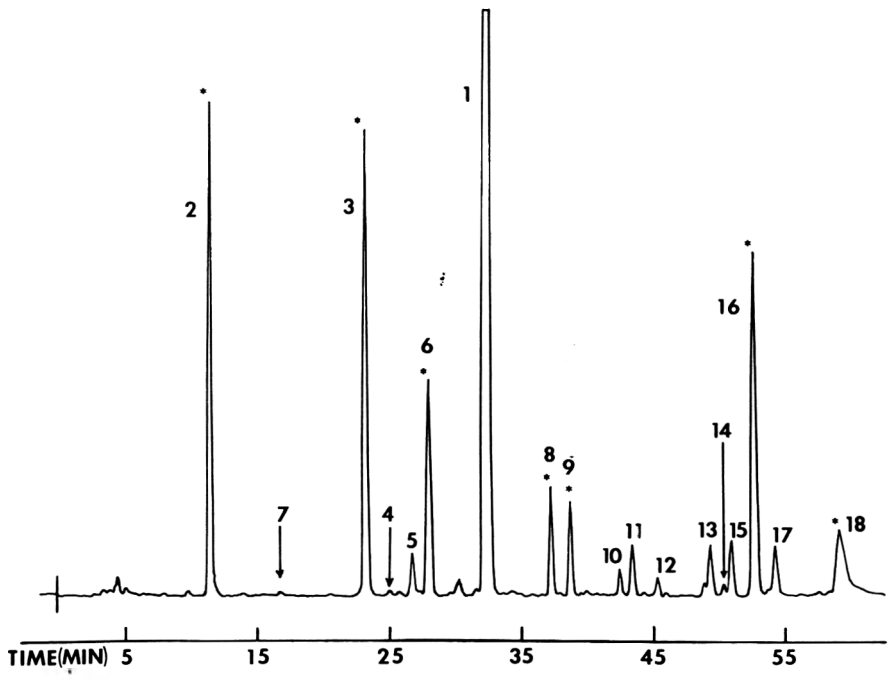


Figure 1. Normal phase HPLC analysis of concentrated impurities from methoxychlor. Conditions: hexane-CH_2Cl_2, 10-40% CH_2Cl_2 (30 min), 40-100% CH_2Cl_2 (20 min). Flow rate, 1 mL/min using Si-10, 4 mm × 30 cm column. Compounds isolated for NMR study indicated by asterisk (semi-preparative column). See Table 2 for identification.

benzene in the presence of catalytic $SnCl_4$, a 3:1 mixture of **21**:**22** is formed as characterized by GC/MS, HPLC, and ^{1}Hmr of the mixture (13).

Condensation Products of 2,2,2-Trichloro-1-(p-methoxyphenyl)ethanol, 13, with DMDT, 1, and o,p'-DMDT, 3

(a) *Impurity* **9**, *reaction of* **13** *with o,p'-DMDT,* **3**.—Boron trifluoride etherate (2.0 g, 0.015 mol) was added to a stirred mixture of carbinol, **13** (14) (1.5 g, 6 mmol), and *o,p'*-DMDT, **3** (1.72 g, 5 mmol) in 50 mL dry benzene, which was then refluxed under nitrogen for 5 h. The reaction mixture was worked up by pouring into water and extracting with several portions of ethyl ether. The crude product was chromatographed over silica gel using pentane-benzene (1 + 1) as eluant to give 2 g condensation product **9** (70%). On repeated recrystallization from methanol, **9** (1.7 g) had mp 80–84°C, reflecting contamination by 3–5% *o,p'*-DMDT, **3** (HPLC). Therefore, the structure was confirmed by 250 MHz ^{1}Hmr (see Table 2), by M^+ at m/e = 580 (isotope pattern indicated 6 Cl atoms), and by conversion to the alkadiene, **25**, by dehydrochlorination.

(b) *Dehydrochlorination of condensation product* **9**.—Compound **9** (0.5 g, 0.9 mmol) was refluxed for 2 h with KOH (0.25 g) in 50 mL ethanol. The mixture was cooled to room temperature, and the solvent was removed. The residual oil was dissolved in methylene chloride, washed exhaustively with water, and evaporated. On recrystallization from methanol-water, **25** was obtained as white crystals (0.350 g), mp 108°C; $M^{+\cdot}$ at m/e 508. Analytically calculated for $C_{25}H_{20}O_3Cl_4$: C, 58.84; H, 3.91; found: C, 58.57; H, 3.92. Assignment of 250 MHz ^{1}Hmr spectrum is given in Table 2.

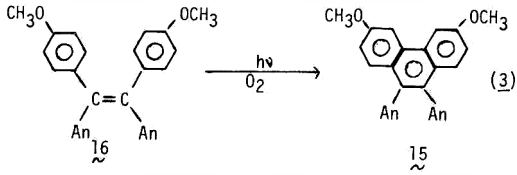
(c) *Impurity* **8**, *reaction of carbinol,* **13**, *with o,p'-DMDT,* **3**.—Employing the same concentration of reactants as in (a) above but with DMDT, **1**, as substrate, extremely slow condensation took place to give **8**. After prolonged reflux resulting in about 20% conversion, purification by repeated chromatography on silica gel using pentane-benzene (1 + 1) gave 0.150 g of a clear solid, mp 130–140°C, containing 3–5% DMDT as the sole impurity (254 nm detection, normal phase HPLC conditions as in Figure 1); $M^{+\cdot}$ at m/e 580 (containing 6 Cl atoms); ^{1}Hmr 250 MHz in $CDCl_3$ given in Table 2.

When **8** was reacted with KOH in ethanol as above, the alkadiene formed had exactly the same HPLC retention time (normal and reverse

phase) as the product from dehydrochlorination of **9.** Moreover the *same* high field PMR spectrum was obtained for both dienes. Therefore, the two m/e 580 compounds are diastereomers. See reference 13 for a complete discussion of the carbocation chemistry involved.

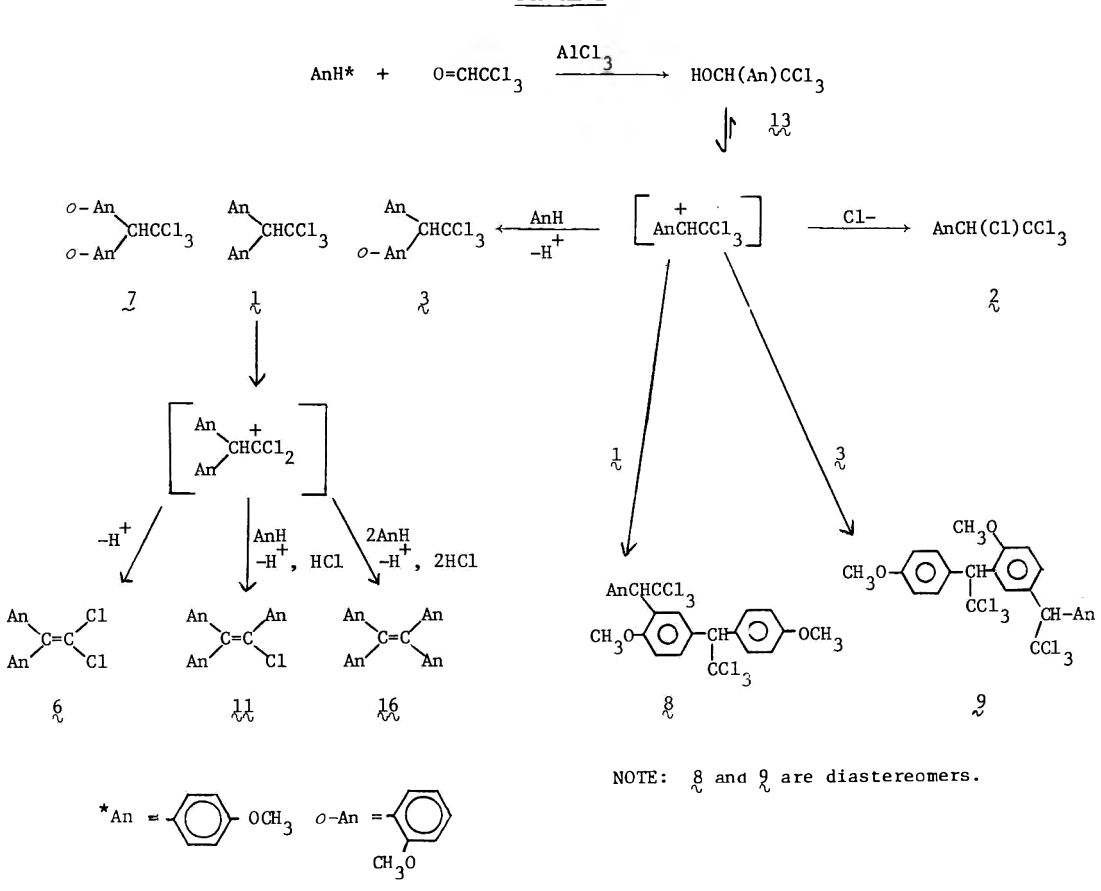
Results and Discussion

After removal of the major *p,p'*-isomer of DMDT, **1,** by crystallization, analysis of the impurity residue by normal phase HPLC gave a chromatogram in which nearly 40 components can be distinguished, Figure 1, Table 1. Relative intensities depend on the 254 nm extinction coefficient; however, sensitivity and resolution compare closely with the overall results by gas chromatography (3). The major components are cleanly separated by both techniques. Higher molecular weight components, with corresponding long retention times, show evidence of tailing by gas chromatography. In contrast, 1,1,2,2-tetrakis(*p*-methoxyphenyl)ethene, **16,** is cleanly eluted by HPLC. Light and air convert **16** to 3,6-dimethoxy-9,10-bis(*p*-methoxyphenyl)phenanthrene, **15,** equation *3* (**4**).



In our hands, **14** and **15** do not elute on GC capillary columns (programming to 260°C). By HPLC, these phenanthrenes are separated from both of the tetra-anisylethylene isomers. The m/e 452 isomer, **17,** with longer retention time has the single *o*-methoxy group. It corresponds to the m/e 452 isomer with longest retention time detected in GC/MS analysis. Both **16** and **17** have now been synthesized and confirm previous mass spectrometric identification. Compound **11,** "chlorotrianisene," is important as a potent synthetic non-steroidal pro-estrogen available commercially under the trade name TACE. It contaminates DMDT as the result of an over-arylation reaction as shown in Scheme 1.

SCHEME 1



NOTE: 8 and 9 are diastereomers.

Table 3. Estimation of concentrations (%) of principal impurities in technical methoxychlor based on HPLC

Compound[a]	Technical (as received)	Impurity conc.	25% Emulsifiable[b] conc.
2	1.32	1.73	0.82
3	4.06	4.03	2.97
6	0.46	0.39	0.58
8	0.73	0.48	0.50
9	0.40	0.40	0.69
16	0.53	0.50	0.18
	Minor Constituents[c]		
11		0.050	
17		0.060	

[a] Reference Table 1 for structures.
[b] Expressed as percentage of nominal technical pesticide content.
[c] Measured in impurity concentrate.

The absence of the major components **8** and **9** from a GC trace of methoxychlor impurities illustrates the complementary value of HPLC in the technical methoxychlor analysis. These 2 secondary condensation products arise from further attack on DMDT, **1**, and its *o,p'*-isomer, **3**, respectively. In Scheme 1, a brief outline of the relevant carbonium ion chemistry is given. Combining the high resolution of normal phase and a semi-preparative Si-10 column (8 mm × 50 cm), 1–2 mg of **8** and **9** were obtained separately. The initially unknown structures were assigned by ^{1}H NMR (accumulated FT spectra) and by probe CI and EI mass spectrometry. For these involatile minor components, HPLC proved essential. More generally, all 7 compounds starred in Figure 1 were collected and ^{1}H NMR and MS data were obtained. Comparison of the GC/MS retention times of the isolated fractions with retention times of the methoxychlor impurity could then be made. The procedure facilitated rapid preliminary assignment of mixtures where capillary GC and HPLC were the only techniques with sufficient resolution.

For the major components, authentic samples were synthesized for confirmation and quantitation. The concentrations of the major high MW constituents based on calibration at 254 nm are given in Table 3. The percentages given for the concentrated sample are back-calculated on the basis of DMDT, **1**, removed during recrystallization. For the formulation, the *total* technical DMDT is 25% w/w, and impurity concentrations in the CH_2Cl_2 solution of the concentrate are related to that content. Note that some changes in concentration (m/e 580, material **8**) occur on recrystallization in our hands. Direct assay may be preferable for these impurities. The relatively low values (beyond ±10% range) for *p,p'*-DMDT, **1**, *o,p'*-DMDT, **3**, and TAE, **16**, in the 25% EC were confirmed in duplicate experiments. Such variability may reflect manufacturing conditions, or loss of reactive impurities during handling. Accordingly, the range of values in Table 3 provides a guideline but *not* an absolute standard for methoxychlor samples in general.

The major non-chlorinated impurity **16**, TAE, may also be analyzed spectrophotometrically as the blue dication employing a Br_2–acetic acid reagent (4). Assay at 540 nm gave 5000 ppm of **16**, in agreement with HPLC values for DMDT. Based on GC analysis (5), we previously estimated TACE, **11**, at 100 ppm (min.), somewhat less than the HPLC value of 0.050%. Since the active dose of TACE in rats is 18 μg (9), measurement of **11** has interested us in the on-going monitoring of DDT pesticides for estrogenic activity (10).

Reverse phase operation employing a Varian MCH-10 column with water–methanol elution gave more reproducible retention times. Slight deactivation by water sharply reduces normal phase resolution of the impurities. However, reverse phase involves significant sacrifice in resolution as well (Figure 2). Even DMDT, **1**, is not separated from its *o,p'*-isomer, **3**; the m/e 580 materials, **8** and **9**, appear as a single peak; and the pro-estrogen TACE, **11**, is obscured by the dechlorinated analog trianisylethylene, MW 346, **12**. In the reverse phase chromatogram, clean separation of mono-demethylated DMDT, **18**, from the remaining components is achieved. Comparison of the impurities mixture (Figure 2) with the direct methylene chloride extract of 10% methoxychlor dust (Figure 3) illustrates the rapid

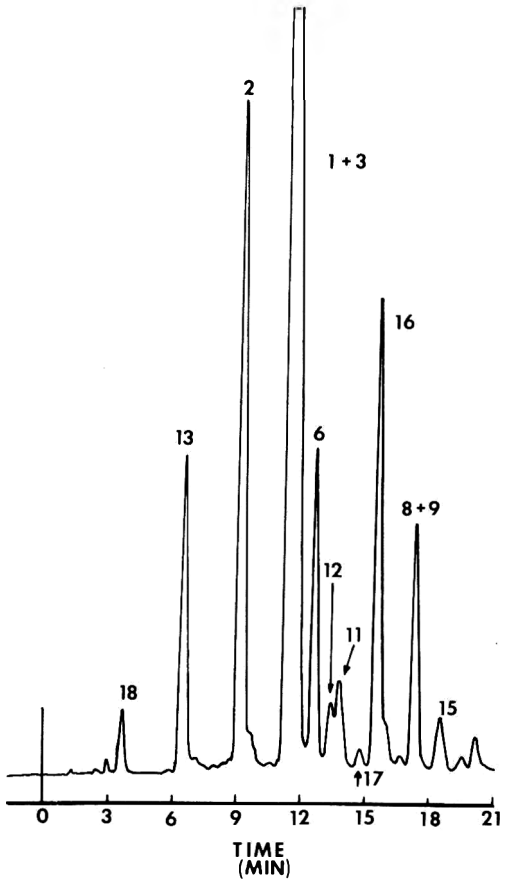


Figure 2. Reverse phase HPLC analysis of impurities concentrated from technical methoxychlor. Conditions: 70% methanol-water to 100% methanol (20 min). Flow rate 1.2 mL/min using MCH-10, 4 mm × 30 cm column. Temperature 30°C; UV detection (254 nm). See Table 2 for identification.

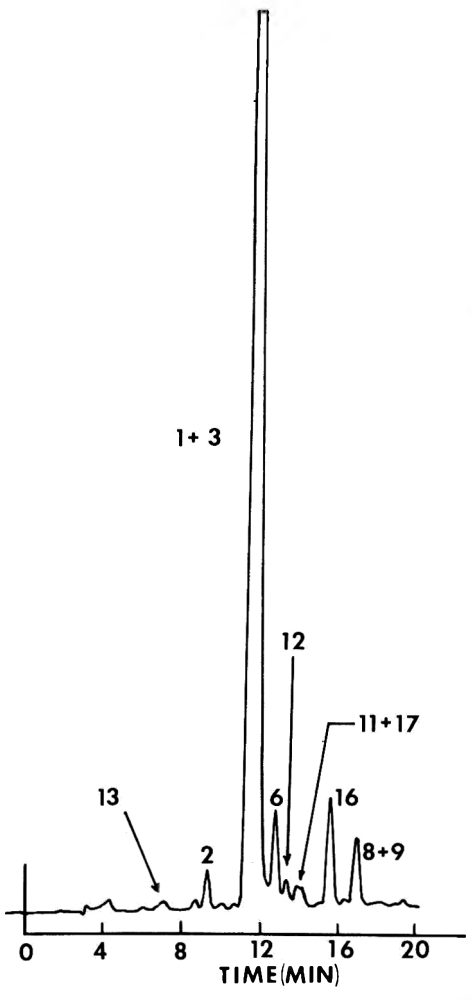


Figure 3. Reverse phase HPLC analysis of CH_2Cl_2 extract of 10% wettable powder methoxychlor formulation. Conditions as in Figure 2.

screening of commercial samples by reverse phase HPLC. However, aromatic solvents in the 25% EC Greenleaf interfere with phenol, **18**, in reverse phase operation (Figure 4). However, in normal phase operation, these xylenes also elute at short retention times, while **13** and **18** are retained longer (cf Figure 1).

In the environment, photolysis and photo-oxidation produce complex mixtures of polar and high molecular weight components. Figure 5 shows the reverse phase chromatogram of methoxychlor impurities exposed to sunlight and air as a thin film, and then redissolved in CH_2Cl_2. Analytically, the expected large number of new components is the most significant factor. However, several trends can be identified by comparison with the original sample, Figure 2. The alkenes, DMDE **6** and *o,p'*-DMDE **4**, are now enhanced; there is a loss of TAE, **16**, and an increase in the corresponding phenanthrene, **15**. The involatile condensed components 8 and 9 persist. As noted above, methoxychlor sample history alters the relative composition of the contaminants.

Photo-oxidation also introduces oxygenated products whose separation requires a less polar solvent program. Figure 6 shows better separation of the possible polar components indicated in Figure 5. Anisic acid (A), and *p,p'*-dimethoxybenzophenone (D) are confirmed in the oxidation mixture using the conditions indicated for the standards.

In the biosphere, DDT pesticides are extremely sensitive to reductively induced rearrangement

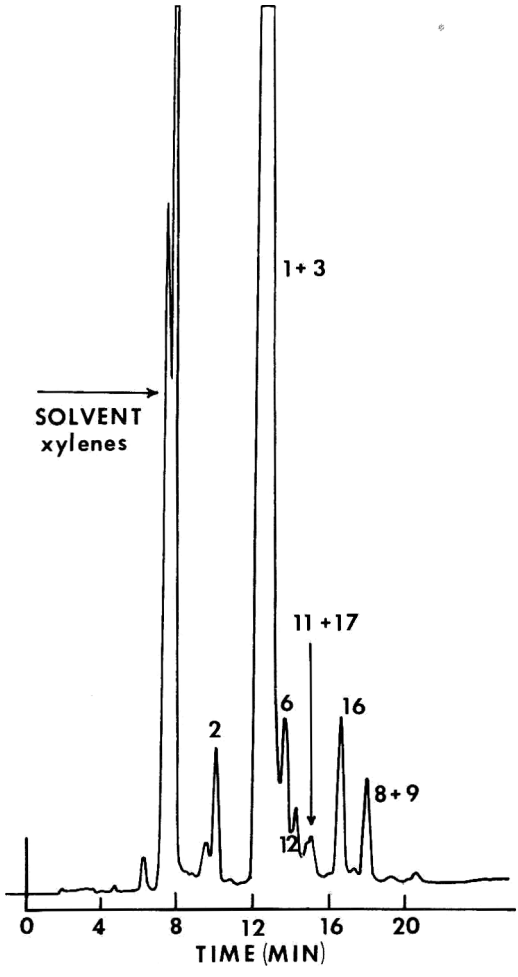


Figure 4. Reverse phase HPLC analysis of 25% emulsifiable concentrate methoxychlor formulation. Conditions as in Figure 2.

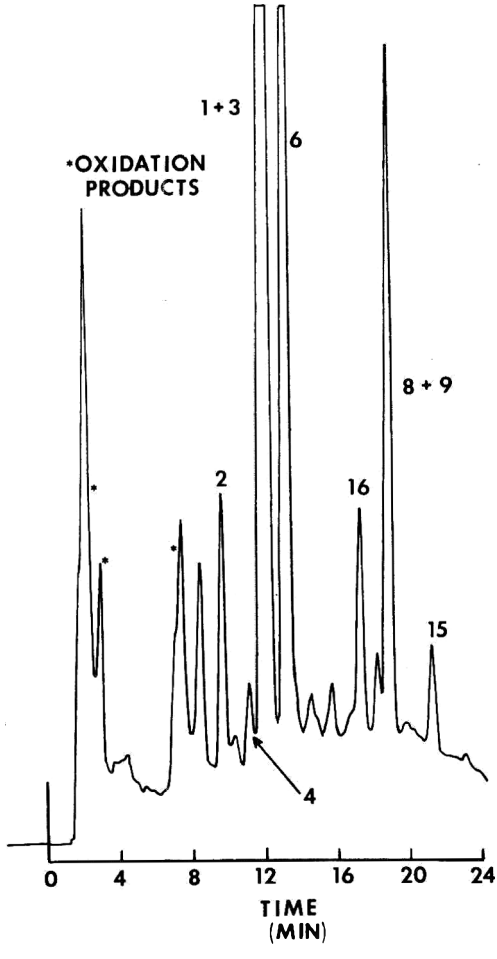
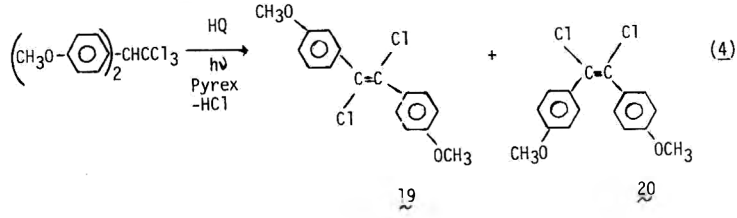


Figure 5. Reverse phase HPLC analysis of methoxychlor impurities exposed to sunlight as a thin film. Conditions as in Figure 2.

and chlorine loss. We have applied both GC/MS and HPLC analysis to the sunlight-sensitized anaerobic reduction of methoxychlor by a donor (hydroquinone) in CH_3CN (11). The major products of interest were the (E)- and (Z)-1,2-dichloro-1,2-di[*p*-methoxyphenyl]ethenes, **19** and **20**, equation 4. These 2 stilbenes are isomers of *p,p'*-DMDE, **6**, MW 308. When the corresponding stilbenes and alkene from the *o,p'*-DMDT isomer, **3**, are considered a total of six MW 308 isomers are all potentially reaction products. Figure 7 shows that both GC and normal phase HPLC provide a reasonable separation of these isomers.



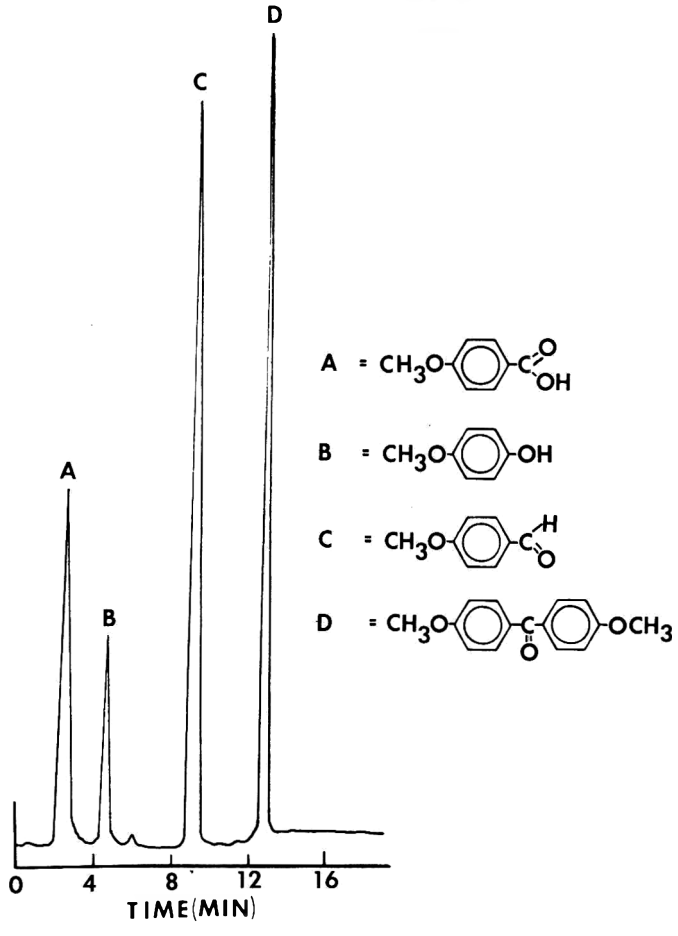
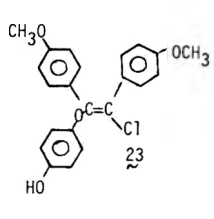


Figure 6. Reverse phase separation of potential polar photo-oxidation products formed from methoxychlor. Conditions: 45% methanol-water (0-5 min) to 100% methanol (5-10 min). Flow rates 1 mL/min (0-5 min) to 1.5 mL/min (5-10 min). MCH-10 column and UV detection (254 nm).

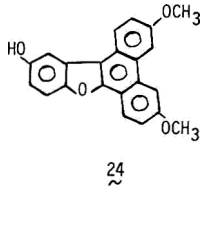
Reaction products from the reduction of either 1 or 3 were analyzed by both retention time and co-injection of authentic material. Figure 8 shows the complexity of the actual reaction mixture from DMDT, **1**. Capillary GC was a necessary confirmatory analytical technique. Both the Varian 3700 GC (FID detection) and the Finnigan 3300 GC/MS (ion chromatogram) (Figure 9) have been used in conjunction with HPLC to obtain all the assignments that are shown. Quantitation of the (E)-stilbene (**19**), a known pro-estrogen active at 200 γ (12), was undertaken by GC (2.49% of product) and HPLC (3.0%). In each case, linear calibration of the detector was made using authentic material. It should be noted that several mono-hydro derivatives, MW 274, are observed.

We have carefully examined the methoxychlor impurity concentrate and detect a low concentration (10 ppm) of (E)-stilbene (**19**) using the Finnigan GC/MS system. However the generation of the stilbenes by reduction may elevate their concentration in environmental samples.

In spite of the apparent clear picture presented by these techniques, the principal products (representing 60% of the technical material) were the conjugates shown in Figure 10 obtained using reverse phase operation. Triarylethylene,



23



24

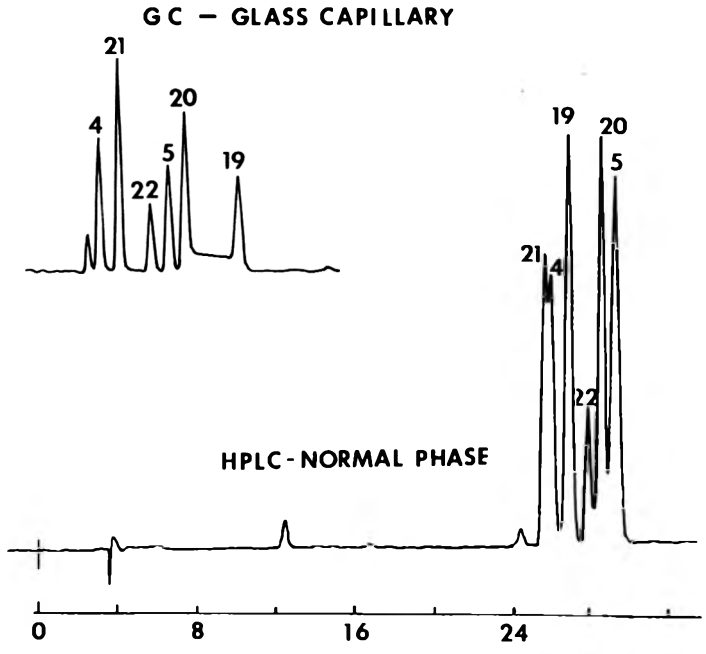


Figure 7. Separation of standard mixture of m/e = 308 isomers formed from methoxychlor. Conditions: (1) gas chromatography (inset): 60-240°C at 10°C/min. Temperature: injector 260°C, FID 350°C. (2) Normal phase HPLC: Hexane-CH_2Cl_2, 10-40% CH_2Cl_2 (30 min), 40-100% CH_2Cl_2 (10 min). Identification reference Table 4. Note: Elution times refer only to HPLC.

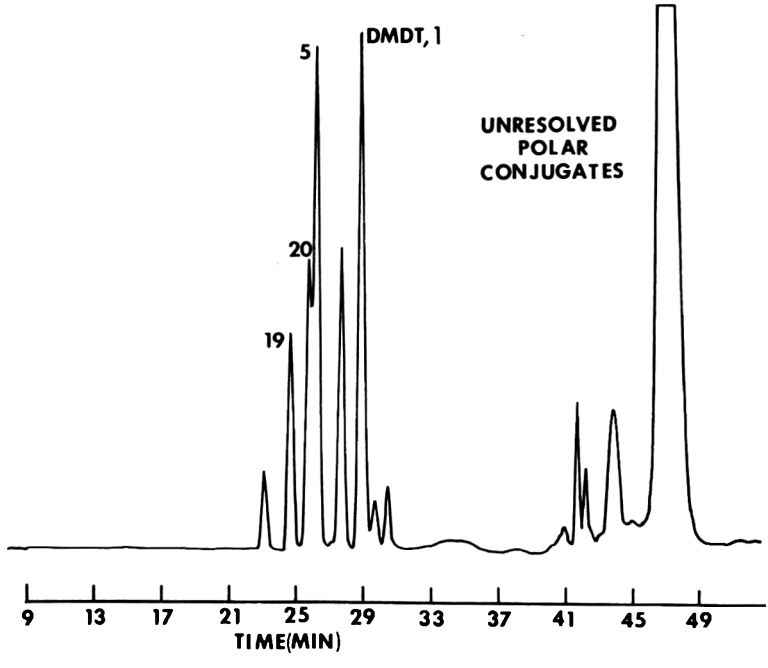


Figure 8. Normal phase HPLC analysis of products formed in reductive decomposition of methoxychlor. Conditions as in Figure 1. Identification reference Tables 2 and 4.

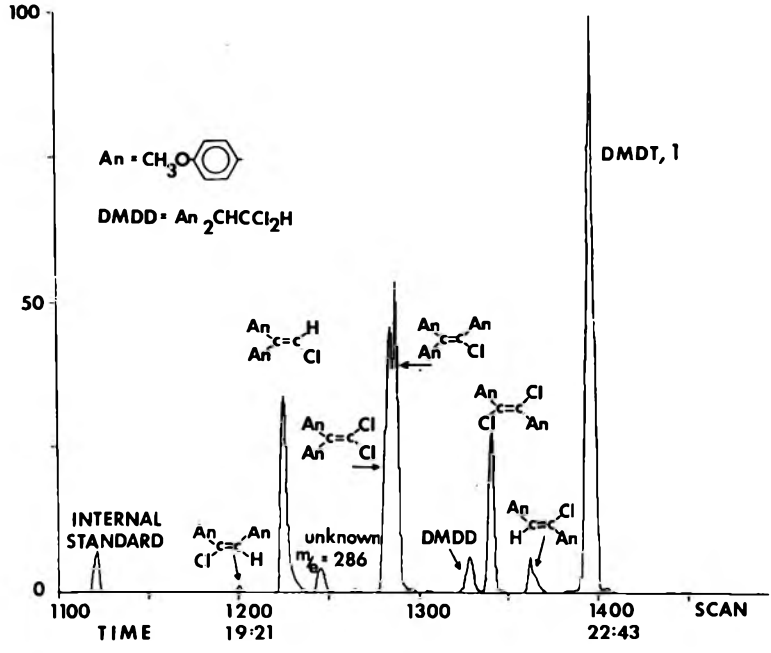


Figure 9. GC/MS analysis of methoxychlor reductive degradation. Ion chromatogram of methoxychlor olefin and stilbene mass region. Finnigan 3300-INCOS Data System output (flex capillary column).

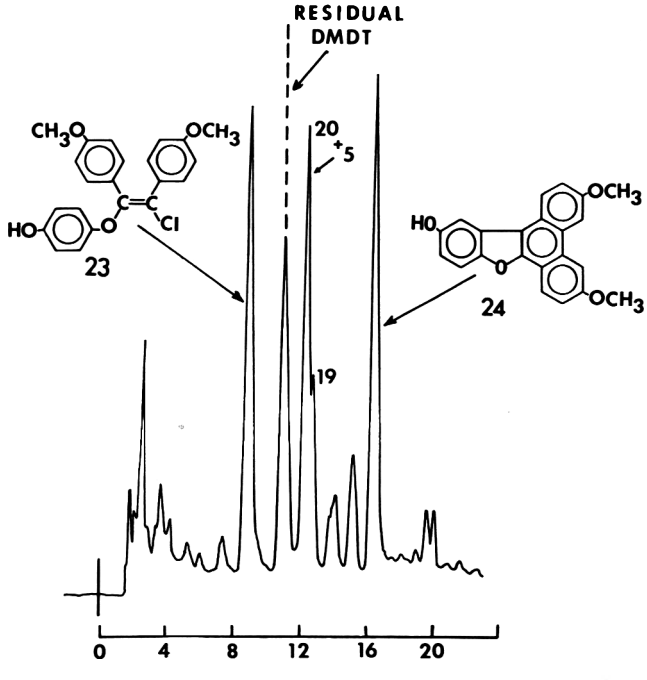
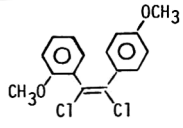
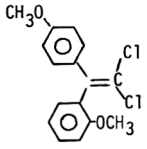
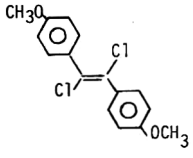
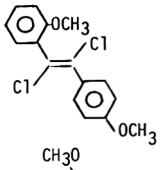
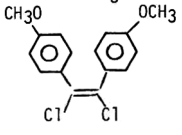
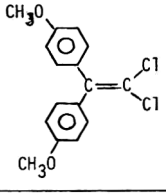


Figure 10. Reverse phase HPLC analysis of hydroquinone-induced methoxychlor reductive photodecomposition. Conditions as in Figure 1. Identification reference Table 4.

Table 4. Common isomeric m/e 308 impurities in reductively degraded methoxychlor

Structure	Designation
	21
	4
	19
	22
	20
	6

23, is detected by GC at a retention time 75 min (260°C), but compound 24 is not eluted from low temperature capillary columns. The utility of reverse phase HPLC for high molecular weight pesticide conjugates is clearly demonstrated.

Summary

In the analysis of technical methoxychlor and related environmental samples, HPLC provides significant complementary data when used in conjunction with capillary GC/MS studies. Higher molecular weight components are detected with improved separation, and a substantial impurity fraction (1% of condensed components) is uniquely revealed by HPLC. Degradation of methoxychlor generates related compounds that are isomers of the initial impurities. These potential components are readily separated by combined use of GC and HPLC analysis.

Acknowledgments

The Institute of Ocean Sciences, Canadian Federal Ministry of Fisheries and Oceans, Pat Bay, Sidney, British Columbia, is thanked for use of the Finnigan 3300 GC/MS-INCOS data system. Helpful discussions with W. J. Cretney of the Institute are gratefully acknowledged. Financial support by a strategic environmental toxicology grant from the National Science and Engineering Research Council of Canada is acknowledged.

References

(1) (a) Metcalf, R. L. (1974) in *The Future for Insecticides, Needs and Prospects*, R. L. Metcalf & J. J. McKelvey, Jr (Eds), Vol. 6, Wiley, New York, NY, pp. 223, 285
(b) Gardner, D. R., & Bailey, J. R. (1975) *Methoxychlor: Effects on Environmental Quality*, National Research Council of Canada, NRCC 14/02, Ottawa, Ontario, Canada
(2) Technical Report: Control of Black Flies in the Athabasca River (1980), W. O. Haufe & G. C. R. Croome (Eds), Pollution Control Division, Alberta Environment, Edmonton, Alberta, Canada, pp. 39–93
(3) Lamoureux, C. H., & Feil, V. J. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1007–1037
(4) Mitchell, R. H., Mazuch, L., Shell, B., & West, P. R. (1978) *Can. J. Chem.* **56**, 1246–1252
(5) Mitchell, R. H., & West, P. R. (1978) *Chem. Ind.* 581
(6) Kapoor, I. P., Metcalf, R. L., Nystrom, R. F., & Sangha, B. K. (1970) *J. Agric. Food Chem.* **18**, 1145–1152
(7) Zepp, R. G., Wolfe, N. L., Gordon, J. A., & Fincher, R. C. (1976) *J. Agric. Food Chem.* **24**, 727–733
(8) Curtin, D. Y., & Flynn, E. W. (1959) *J. Am. Chem. Soc.* **81**, 4717–4728
(9) Shelton, R. S., et al. (1953) *J. Am. Chem. Soc.* **75**, 5491–5495
(10) McLachlan, J. A. (Ed.) (1980) *Estrogens in the Environment, Developments in Toxicology and Environmental Science*, Vol. 5, Elsevier-North Holland, New York, NY
(11) West, P. R., Chaudhary, S. K., & Mitchell, R. H. (1981) unpublished results
(12) Oki, M. (1953) *Nippon Kagakukai Bull.* **26**, 37–39
(13) West, P. R., Chaudhary, S. K., & Mitchell, R. H. (1982) *J. Org. Chem.* (submitted)
(14) Goodford, P. J., et al. (1976) *J. Med. Chem.* **19**, 1239

SYMPOSIUM ON INFANT FORMULA REGULATION AND INFANT FOOD PROBLEMS

95th Annual Meeting of the AOAC, October 1981

At the 95th Annual Meeting of AOAC, October 1981, representatives of infant formula producers and staff scientists of the Food and Drug Administration joined to present current information on the status of infant formulas and proposed regulatory initiatives. Increased concern about infant formulas was the result of an unfortunate incident in which a chloride deficiency in one manufacturer's products presented a hazard to infant health. The outcome was passage of the Infant Formula Act and FDA proposals for quality control regulations. The papers given at the Symposium are presented below. Several of the original manuscripts have been revised in light of differences between the proposed and the final regulation. The information contained in these papers should be of considerable value in understanding the present status of infant formulas and their regulation.

M. J. DEUTSCH

Food and Drug Administration
Division of Nutrition
Washington, DC 20204

The Infant Formula Act of 1980

RAYMOND E. NEWBERRY
Food and Drug Administration, Division of Regulatory Guidance, Washington, DC 20204

The Infant Formula Act of 1980 is one of the most specific and detailed acts ever passed by Congress. It establishes minimum nutrient requirements, defines adulteration, provides for establishing nutrient and quality control procedures, prescribes recall procedures, and specifies inspection requirements.

The passage of legislation often represents a reaction to an event or crisis, rather than a planned occurrence in anticipation of a future problem. This is understandable; often legislators can obtain unity only when some tragedy or disaster strikes, or if a Congressman championing a cause can convince his colleagues of the importance of that cause. The history of United States food and drug laws is a good illustration. Meat preserved with formaldehyde killed a number of soldiers; sulfanilamide dissolved in a toxic solution killed a number of children; thalidomide resulted in a number of birth deformities. All of these incidents caused reactions among the Congress that resulted in the passage of legislation.

Similarly, in 1979, the manufacturers of a popular infant formula reacted to public outcry against too much salt in the diet of infants by reducing the salt in their formula. Unfortunately, this reaction resulted in a formula that was deficient in chloride, and this chloride deficiency had adverse effects on the health of the infants who were fed the formula. The resulting publicity caused a reaction among members of Congress that led to the Infant Formula Act of 1980, one of the most specific and detailed acts ever passed by Congress.

The Act defines infant formula as a food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk. Basically, this Act establishes minimum nutrient requirements (as well as some maximum requirements) for infant formulas and gives FDA authority to establish requirements for nutritional and quality control, record keeping, notification and recall procedures.

This Act specifies that an infant formula is adulterated if it meets any of the 3 following conditions: (1) If it fails to provide nutrients as required, (2) if it fails to meet the nutrient quality factors required by regulation, or (3) if the processing is not in compliance with appropriate quality control procedures or record retention requirements as prescribed by regulation.

The Act spells out in detail the nutritional requirements for infant formulas, yet provides for the revision of these requirements by regulation as needed. One may wonder why the specific detailed list of nutrients is a permanent part of the Act itself when it may be changed by regulation. This means that, if at some future date the list in the Act and the list in the regulations significantly disagree, the regulations would take precedence over the Act itself, since revising the nutrient list is permitted by the same Act.

The Act provides for the issuance of regulations to establish nutrient and quality control procedures for infant formula processing. Although the Act merely states that such regulations "may" be established, what follows really says, in effect, that such regulations "will" or "shall" be established. In stating that regulations may be established for quality control procedures the Act goes on to require that these regulations assure that appropriate nutrients are provided and tested for, and that testing records are maintained and retained.

As an interim measure, until quality control procedures become effective as a regulation, the Act requires all infant formula manufacturers to notify FDA every 90 days that each infant formula manufactured provides the nutrients required by the Act. For new infant formulas, the Act requires the manufacturer to notify FDA (at least 90 days before distribution) whether the new product meets the nutrient and quality factor requirements and whether the processing meets the quality control procedures required. The Act also requires that FDA be notified before any changes are made in the formula or processing which the manufacturer determines may affect whether the formula is adulterated.

If an infant formula manufacturer has knowledge which reasonably supports the conclusion that his product has left his control and may not provide the nutrients required or may be otherwise adulterated or misbranded and, if adulterated, presents a health hazard, he is re-

Presented at the Symposium on Infant Formula Regulation and Infant Food Problems, 95th Annual Meeting of the AOAC, Oct. 19–22, at Washington, DC.

quired to notify FDA of the problem.

Whenever a manufacturer makes an infant formula recall, he is required to carry out the recall in the manner prescribed by regulation. The manufacturer making the recall is required to report all recall implementation actions to FDA every 2 weeks until it is terminated. FDA, on the other hand, is required to review the firm's recall actions every 15 days to determine whether the actions meet the prescribed requirements.

The Act states that FDA *"shall,"* by regulations, prescribe the scope and extent of infant formula recalls necessary and appropriate for the degree of hazard involved. The infant formula manufacturer must prepare records concerning distribution of his products in order to effect and monitor recalls and to keep such records for 2 years. If FDA determines that appropriate records are not being prepared or maintained, it may prescribe by regulation what records need to be maintained and how they should be retained.

The Act exempts what are commonly known as "medical foods" from the requirements of the Infant Formula Act except for (1) providing notice to FDA whenever the manufacturer has knowledge that his product is adulterated or misbranded and presents a risk to health and (2) compliance with the recall regulations promulgated by FDA. In this respect the Act also permits FDA to grant and withdraw exemptions from the adulteration provisions and the requirement for reporting that "medical food" infant formulas contain the nutrients required.

The Act extends the factory inspection section to permit FDA representatives to have access to records and test results necessary to confirm compliance with the new Act.

The Prohibited Acts section has been revised to include (1) failure to provide the notices to FDA as required, (2) failure to report recall implementation procedures at least every 14 days, (3) failure to meet appropriate recall requirements prescribed by regulation, (4) failure to maintain, permit access to, or permit copying of records, and (5) prohibition of the disclosure of trade secrets to unauthorized persons.

The Secretary of Health and Human Services is required by the Act to prepare reports and submit them to the Congress on the long-term health effects on infants of hypochloremic metabolic alkalosis, the effects of labeling on infant nutrition and proper use of formula products, and the issues surrounding export of infant formulas.

Infant Formula Label Review

ROGER W. BARNES
Food and Drug Administration, Washington, DC 20204

The FDA review of infant formula labeling was conducted by a multi-disciplinary team composed of physicians, scientists, and experts in regulations and labeling. The review followed 2 tracks: the development of a general position of what would be necessary if we started with a blank label; and the purchase of many formulas and a critical review of information on the labels. An important part of the review was determining what information was available in the literature and using this information in assessing current practices and the effectiveness of present labeling. The review was divided into several major topics: introduction, definitions, historical perspectives, current regulatory situation, effectiveness of labeling, label review, other issues, and departmental initiatives. The labels review included product attributes, shelf life, retail and home storage instructions, and consumer preparation and use.

The Food and Drug Administration (FDA) was required by the Infant Formula Act of 1980 to review infant formula labeling. The review was conducted by a multi-disciplinary team composed of physicians, scientists, and experts in regulations and labeling. The team was drawn together in late October 1980, and the reports of Congressional committees and the Infant Formula Act were carefully reviewed to identify key points to be addressed. The review had a very high priority and it was decided that it would be both rigorous and scholarly. The outline for the report was prepared and it was circulated to ensure that the salient points were identified.

Once the key points were agreed on, the general format was to discuss the significance of each point, the present Federal requirements relating to that point, the current industry practice or how the industry was labeling infant formulas, the effectiveness of the regulation(s) or industry practice in addressing the point, and a summary of the discussion.

Two essentially different tracks were followed in the review of these points. The first was to think about the point and to come to a general position of what would be necessary if we were to start with a blank label. This approach was unfettered by constraints of regulation or current labeling practices.

The second track involved the purchase of many infant formulas and a critical review of the information on the label, including its format, language, and potential impact on the user. This track yielded considerable useful information and disclosed points that we believed required some changes, almost irrespective of any conclusions reached by reviewers following the first track.

The different sections or elements of the review of each key point were assigned to the several reviewers. To the extent possible, a reviewer was assigned only one section of each point. Thus a reviewer looking at current industry practices was responsible for reviewing and preparing only that section. There was, however, close collaboration between the reviewers so that necessary expertise was available in order to make each of the sections complete and balanced. As an example, the reviewer writing the *Significance* section frequently consulted with other reviewers for medical, scientific, or regulatory input.

An important part of the review was related to determining what information was available in the literature and using this knowledge in assessing current industry practices and the effectiveness of present or potential labeling requirements. Unfortunately, there was not a great deal of pertinent literature available. What was available—for example, the use of colors or pictograms and reports of injuries associated with misuse or incorrect use of infant formulas—was critically reviewed. In addition to the literature, reports of consumer opinions concerning labeling were reviewed, as were reports by expert groups about problems associated with infant formula labels and the safety and quality of infant formulas.

Sources outside of FDA were also contacted for views regarding the issues identified by the Congressional committees. It is important to note here that the Infant Formula Council stated that the infant formula manufacturers have voluntarily modified and adapted the labeling of infant formula to respond to consumer needs. This approach includes the use of symbols or pictograms to reinforce instructions for proper

Presented at the Symposium on Infant Formula Regulation and Infant Food Problems, 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

0004-5756/82/6506-1474–04$01.00

and sanitary preparation of infant formulas. The industry has also acted to modify or adapt the nutrients and their levels in response to recommendations of authoritative medical and scientific bodies.

The review included only the labels for infant formulas that are generally available and that are intended for use by normal infants. "Special formulas" that are designed to meet the special needs of infants with an inborn error of metabolism or low birth weight or who otherwise have an unusual medical or dietary problem were exempted from pertinent parts of the ingredients and labeling sections of the Infant Formula Act of 1980, and therefore were exempted from the review. The labeling of these formulas is under active review within the Department of Health and Human Services (DHHS).

Breast-feeding is not addressed in the context of labeling, and it is not covered in the report of the review. This is a separate issue which needs consideration in the context of developing scientific answers to the many questions surrounding breast-feeding practices.

The report of the review is divided into several major topics:

1. Introduction

2. Definitions: This topic includes, for example, the terms "infant," "proper use," and "infant nutrition."

3. Historical Perspective: This topic describes the legal authorities and the general philosophical approach to the labeling of consumer products which FDA follows. It was determined that the rules and regulations of FDA comprise the ". . . existing federal requirements for the labeling of infant formula . . ." and that other departments or agencies generally cross-reference these regulations. However, meat-based formulas are subject to dual regulation; both DHHS and the U.S. Department of Agriculture (USDA) regulate these formulas.

4. Current Regulatory Situation: This topic covers the general labeling requirements of FDA and includes the changes brought about by the Infant Formula Act of 1980. The regulations affecting the labeling of infant formulas (21 CFR 105.65) are specifically discussed under this heading.

5. Effectiveness of Labeling: This topic includes a brief discussion of the use of colors and of pictograms. These topics are also covered specifically within the sections of the infant formula label review.

6. Infant Formula Label Review: This topic covers 5 major aspects, some of which have been subdivided for the purposes of the review and the report. Some aspects of the target groups for infant formula labeling and the importance of labeling are discussed below.

7. Other Issues: This topic covers such issues as special formulas, precautionary labeling, and bilingualism.

8. Departmental Initiatives: This topic is a description of efforts that were already underway at the time of enactment of the Infant Formula Act of 1980 or that arose as a result of the Act.

The first 5 topics are essentially self-explanatory, although 2 points should be noted with regard to the historical perspective.

First, the role of labeling is founded upon the premise that consumers can and will make reasonable choices if presented with truthful and accurate labeling. This premise is of key importance. Second, labeling has become even more important as consumers deal less and less with store owners or clerks and rely more on product selection in self-service outlets. Prepackaged food may prevent inspection by the consumer. Food is more likely to contain preservatives and colors, to have a modified or special composition (e.g., low sodium, hypoallergenic food, etc.) or to contain alternative sources of nutrients (e.g., plant proteins). Thus labeling is the single best method of alerting consumers to the facts they need to make decisions on the food they purchase. These 2 points stress the importance for labeling products in a truthful and accurate manner so that they do not mislead or deceive consumers.

With regard to the infant formula label review itself, the review notes that infant formula labels must be designed with the eventual user in mind. They must disclose important information about the product. Examples include the identity of the maker of the product or the one responsible for it, the manner of its use, and limitations or warnings concerning its use. The type and amount of information varies with the audience. For example, prescription drug labeling is highly technical and extensive because of the need to communicate complex and important information to health professionals. Patient information for prescription drugs, on the other hand, is less extensive and less technical because it must adequately communicate with the lay public.

The labels of infant formulas are designed for both professional and lay users, whose needs are different. Thus the types of information needed can lead to problems in presenting adequate in-

formation in the space available. Pediatricians, for instance, are concerned about the nutritional and caloric needs of the infant. Labeling allows the pediatrician to instruct the parents about feeding schedules and the amount of formula to be fed. Parents need to be able to identify the correct formula, to handle and prepare it correctly, and to store it after preparation. Manufacturers can, therefore, face very real problems in presenting the information in a manner readily understood by both audiences in the limited space available.

The review also noted that although several sources of information may be available (e.g., books, pamphlets, instructions by health care professionals, etc.), the label is the single source of information that is readily available and is specific for a particular product. The label provides vital information for product selection and instructions for preparation and handling. Proper and adequate labeling is thus important in promoting the safe use of the infant formula.

The first specific element of labeling is "Product Attributes." Two attributes are identified: the base (e.g., milk-based or non-milk-based) and the form (e.g., ready-to-use, concentrated liquid, or powder).

A variety of protein, carbohydrate, and fat sources are used as base constituents in infant formulas. Their identification has a health significance because of possible hypersensitivities or intolerances. The variety of bases used provides a range of alternatives for meeting the individual needs of most affected infants. The present practices and regulations are believed to be generally effective in identifying the base constituents.

Several physical forms of infant formulas are available. The choice of the form usually depends more on economic and convenience factors than on health concerns because nutritional and safety standards apply to all infant formulas intended for use by normal infants. The present regulations require a statement of identity of the product. Because of this regulation, and current industry practice, the form of the product is identified on the principal display panel. Ready-to-use formulas carry a statement that water should not be added. Concentrated liquids and powders must be diluted with water to prepare a formula for the infant. Some manufacturers have adopted similar or identical pictorial symbols for use on the principal display panel to illustrate the need to add water to a measured amount of concentrated liquid or powder. In addition, some manufacturers have also adopted a similar three-step pictorial symbol showing product preparation. The simple presence of pictograms may serve to alert parents that there are differences in form and cause them to look more closely at the container they purchase. Although some manufacturers use color coding for product differentiation, the practice is limited and the colors vary with the company. Color is frequently used, for example, to distinguish formulas fortified with iron from those that are not iron-fortified.

The second element examined was shelf life. Present regulations do not require open date labeling of infant formulas. Many manufacturers, however, have voluntarily adopted the practice of placing "use by" or "use before" dates on the top of containers. Open date labeling of infant formulas was one of the initiatives included in efforts to revise infant formula regulations before the enactment of the Infant Formula Act. Such dating is viewed as an important aspect of labeling, and its use is growing.

The third element was retail and home storage instructions. There are no requirements for storage information on the label of an infant formula. The industry generally includes some storage information on the cartons or cases of infant formula, but this is directed to shippers or to retail outlets. Proper storage before and after opening is important in maintaining the safety, nutritional quality, and desirable characteristics of the product, and some information could be useful to consumers.

The next element was consumer preparation and use. This was subdivided into methods of preparation and sanitation, and these 2 issues were individually examined.

It is obvious that the nutritional quality of an infant formula, even though properly manufactured, packaged, and stored, can be adversely affected if the formula is not correctly prepared before feeding. These instructions can range from a simple "shake well" to the more complex instructions for diluting a concentrated formula or powdered product. Sanitation, particularly if the formula is stored after opening and preparation, is also important.

Errors in formula preparation by consumers have been reported as causes of illness. Consistent use of improperly prepared formula (e.g., under- or over-dilution of concentrated or powdered forms or dilution of ready-to-use formulas) would result in improper nutrient densities and caloric intakes. The literature contains data suggesting that errors in dilution are more

common and serious with powdered products than with liquids. Parents tend to use heaping measures of powder. Powders are more difficult to mix so as to avoid lumpiness.

The labels of concentrated and ready-to-use formulas have "shake well" on the direction panel and frequently on the lid as well. This is important for obtaining a good distribution of the product either before feeding or before a portion of a concentrated liquid is measured for dilution.

Some labels employ graphics (e.g., symbols or pictograms) to reinforce narrative instructions. Several companies use a 3-step pictogram showing the use of boiling water, proper measurement, and mixing with a measured amount of formula. A study has been made of the use of pictograms by Spanish-speaking mothers who could not read English. The study showed that the use of dilution symbols in conjunction with a 3-step preparation pictogram was generally understood to mean that water must be added before use. Although the effectiveness of the specific symbols or pictograms varies, there is support for the general effectiveness of graphics in making this important instruction understandable to non-English speaking parents. Whether there is a single best graphic and/or pictogram and whether use of graphics should become an industry requirement remain to be resolved. Graphics may also be used to enhance the sanitary preparation of infant formulas. The majority of labels reviewed included instructions that the formula should be sterilized before feeding.

As previously noted, each element or subelement was discussed in terms of significance, regulations, industry practice, effectiveness (of the regulation or the industry practice to resolve or to avoid the problem), and a summary statement.

The review included consideration of the use of precautionary labeling and of bilingualism. Both of these issues were specifically noted by the Congressional committees. Both are important and received considerable thought and debate during the review.

With regard to precautionary labeling, infants with special medical disorders receive formulas tailored to their medical needs and are closely monitored by their physicians. The parents of these children are familiar with their problems and remain in close contact with the physician. Labeling of formulas for these children is directed, in general, to the physician. During the review there were discussions of the problems which may occur if improper formulas are used in feeding normal infants. These problems generally express themselves as nonspecific symptoms such as colic, diarrhea, vomiting, rash, coughing, and rhinitis. These symptoms may also occur as a result of a cold or some other medical problem not related to the use of infant formula. If parents assumed that these symptoms were due to the formula and changed it without checking with the pediatrician, they could complicate the condition and delay initiation of proper treatment and care. Other symptoms, such as poor appetite, slow growth rate, severe diarrhea, abdominal pain, and pulmonary problems, may also be due to causes other than the formula. Inclusion of precautionary labeling could lead parents to attempt to diagnose and treat children through nonspecific information. The simple statement "use as directed by your physician" and normal parental concern when an infant is ill should not be set aside by nonspecific labeling of food.

With regard to bilingualism, there have been suggestions that all, or particular portions of, infant formula labels be printed in Spanish. The review noted that the issues involved in the question revolve around literacy in any language. USDA has noted that the population of its special Supplemental Food Program for Women, Infants, and Children is a low-income group in which poor education and low literacy are fairly common. USDA has expressed interest in dilution symbols or pictograms to assist participants in its programs in understanding the information on the label. Some manufacturers supply Spanish language brochures for infant formulas. Some manufacturers use some Spanish on the labels of their products. As previously noted, many manufacturers employ dilution symbols and/or pictograms to highlight some information regarding preparation of infant formulas.

It is recognized that improved labeling is not a panacea for solving problems of improper formula preparation or lack of education among parents. Serious questions about the usefulness or reasonableness of bi- or polylingual labeling are raised by issues such as literacy in any language and space limitations on container labels. Successful use of symbols and pictograms may serve to obviate or to diminish the need for bi- or polylingual labeling.

Nutritional Adequacy and Safety of Infant Formulas

VICTOR P. FRATTALI
Food and Drug Administration, Division of Nutrition, Washington, DC 20204

The Infant Formula Act contains specific requirements for minimum and, in some cases, maximum levels for a list of nutrients that may be revised as warranted by the development of new scientific information. Formulas are required to be manufactured in accordance with quality control procedures to ensure that the safety and nutritional potency of a formula is built into the manufacturing process. A formula that does not provide the minimum level for any required nutrient shall be deemed to be adulterated and may be subject to removal from the market. A manufacturer is required to promptly notify the Secretary of the Department of Health and Human Services of instances whereby a formula does not meet the nutrient requirements, is otherwise adulterated or misbranded, and, as such, presents a risk to human health. There are other provisions in the Act, but these establish that, for infant formulas, nutritional safety is clearly recognized as an integral part of food safety.

The nutritional adequacy and safety of a product are guaranteed when essential nutrients are provided in amounts to promote the maintenance of good health, yet are not present at excessively high levels that can cause an adverse health effect. This is true not only for an infant formula but, in general, for any food. In fact, this concept of nutritional adequacy and safety may be reduced to a simpler term if one regards nutritional adequacy as a component of the overall nutritional safety of a product.

Over the past several years, we have witnessed a general trend in regulatory matters dealing with foods and nutrition which involves the realization and acceptance that the nutritional safety of a food product, including the manner in which it is intended for use, is a critical aspect in assessment of the safety of that product. In past years, food safety involved chiefly assurance of the absence from foods of pathogenic organisms, toxins, filth, and other deleterious substances. In addition to these traditional safety aspects, in recent times the nutritional quality of foods, particularly those that are used for special dietary purposes, has come under careful scrutiny as a public health matter.

Two recent major occurrences illustrate this point. The first involved the use of protein products promoted for weight reduction which culminated in the liquid protein diet fad during 1976 and 1977. This fad resulted in hundreds of illnesses and a number of deaths among individuals who adhered strictly to the extremely low calorie weight reduction regimen. To deal with this problem of nutritional misuse of a food substance, the Food and Drug Administration (FDA) proposed that protein products promoted for weight reduction bear a warning statement cautioning consumers about the potential for serious illness or death associated with very low calorie weight reduction regimens. This proposal for labeling has yet to emerge as a final regulation.

The second major occurrence pertaining to nutritional safety was set in motion with the marketing in 1978 and 1979 of chloride-deficient infant formulas. In contrast to the apparent stalemate in our ability to finalize a regulation for warning labeling of protein products promoted for weight reduction, Congress reacted swiftly by enacting the Infant Formula Act, which was signed into law in September 1980 as an amendment to the Federal Food, Drug, and Cosmetic Act.

Before reviewing some of the legislative requirements for nutritional safety contained in the Infant Formula Act, I wish to emphasize that the full impact of this law is directed toward any food for normal, full-term infants which is represented for special dietary use by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk. Specifically exempted from some of the requirements of the Act, particularly the requirements for nutrient content, is any formula which is represented and labeled for use by an infant who has an inborn error of metabolism or a low birth weight, or who otherwise has an unusual medical problem requiring some extraordinary dietary treatment.

A few manufacturers of infant formula produce virtually all of the milk-based and soy-based products in the United States for the domestic market. This segment of the food industry has been particularly diligent in its attempts to supply the market with safe and nutritious products, because such products usually provide the sole source of nourishment for infants at an extremely

Presented at the Symposium on Infant Formula Regulation and Infant Food Problems, 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

0004-5756/82/6506/1478–04$01.00

critical period for growth and development. Commercial infant formulas have been available since the late 1930s or early 1940s. Over the years, the safety record of these products has been very good. These formulas must not only be nutritionally sound and highly digestible, they must also be free of pathogenic microorganisms and labeled with information that provides the parents with easily understood instructions for their preparation and feeding. Other than the recent problem of the production of formulas deficient in chloride ion, very few incidents have involved serious problems with the nutritional safety of infant formulas. One such problem occurred in 1953 and 1954 when a popular formula was found to be deficient in vitamin B_6 because a heat-sensitive form of the vitamin was destroyed during thermal processing in the manufacture of the product. Commercially prepared formulas marketed since then have been fortified with pyridoxine hydrochloride, a relatively heat-stable form of vitamin B_6.

The chloride insufficiency was an outgrowth of the discussions that have been going on about the relationship between the intake of salt and the development of hypertension. The level of dietary sodium chloride that may contribute to the development of hypertension in the 15–20% of the population with a genetic predisposition to high blood pressure is unknown. Studies in animals show that genetically predisposed rats develop hypertension when fed high levels of sodium chloride. Genetic variations in humans are large, however, and an association between blood pressure and salt intake has not been demonstrated within selected U.S. populations. The average sodium chloride intake in this country is about 10 g per day, with a range of 4–25 g. This average intake of salt is many times the amount needed to provide a nutritionally required quantity of sodium. The normal nutritional requirement for sodium for growth and for unavoidable losses from skin and through execretion is in the range of 0.1–0.2 g of sodium per day, which is equivalent to 0.25–0.5 g of salt per day.

In light of these considerations, the Food and Nutrition Board and other public health organizations believe that sodium chloride intakes of many people in this country are excessive, particularly for that 15–20% of the American population which is at risk for hypertension. Consequently, there is a belief among health professionals that reduction of sodium chloride intake to a level of about 3 g per day would not be harmful for healthy persons, and it may be helpful for the prevention of hypertension in susceptible individuals for whom salt is a permissive factor. Achieving an intake of 3 g of salt per day normally requires elimination of salt in cooking and at the table, since nondiscretionary salt intake in foods amounts to at least 3 g per day.

Given this basic public health position, a rather strong consumer advocacy developed within recent years to reduce the level of sodium chloride in the American diet through such means as eliminating the addition of salt to processed foods. Such pressure has been brought to bear on the infant food industry and, apparently, has had some impact. The extent to which this type of pressure has also had an effect on the infant formula industry is not known for certain. We do know that, in 1978, one of the major manufacturers of soy-based infant formulas, Syntex Laboratories, made formulation changes that reduced the sodium content of their products. Unfortunately, an apparent concomitant consequence was a substantial reduction of the chloride content of their products far below the minimum level for this essential nutrient recommended in 1976 by the Committee on Nutrition of the American Academy of Pediatrics. The 2 affected products were Neo-Mull-Soy, a milk-free, soy isolate formula fortified with vitamins and minerals for use as a milk formula replacement, and Cho-Free, a milk-free and carbohydrate-free formula intended for use by infants with an intolerance to disaccharides or other complex sugars. Neither formula is currently being marketed, since the manufacturer ceased production of both in 1980.

Because food manufacturers, including those who produce infant formulas, are not required to obtain premarket clearance from FDA for new or reformulated products, we were unaware of possible problems with Neo-Mull-Soy and Cho-Free until several incidents involving a serious illness among infants were brought to our attention late in July 1979. Upon notification of Syntex by our San Francisco District Office, we learned that the firm was aware of the reports we had received and, in fact, had received others. Further investigation by the firm revealed that low chloride levels were suspect in all the reported illnesses. On July 31, the Centers for Disease Control notified FDA headquarters that they had identified 26 cases of hypochloremic metabolic alkalosis associated with the chloride-deficient Syntex products. The illness is characterized in infants by constipation, lethar-

gy, loss of appetite, and failure to gain weight and thrive.

On August 1, 1979, Syntex notified FDA that it would recall all stocks of Neo-Mull-Soy and Cho-Free products to the retail level, inform pediatricians and other physicians of the recall, and issue a press release explaining the action to the public. Recall actions were undertaken almost immediately in accordance with established procedures. Unfortunately, misinterpretation of recall instructions within FDA caused a delay in monitoring the effectiveness of the Syntex recall. Consequently, we did not detect occasions when the products, which should have been removed from marketing channels down to the retail level, were still available for sale to the public.

Upon discovery of this problem, the agency began in late October 1979 to undertake an audit to determine the effectiveness of Syntex's recall while informing the company that their recall was not progressing satisfactorily. By this time, however, the entire incident had become the subject of a TV investigative report in Washington and come under the scrutiny of Representative Albert Gore, Jr, from Tennessee who was the parent of a child fed Neo-Mull-Soy. As a result, Congressional hearings were held, several bills were introduced, and, within less than a year, the Infant Formula Act of 1980 was signed into law.

It is estimated that 20,000 infants were taking the 2 chloride-deficient formulas during the year or so that the faulty products were on the market. Several hundred clinically diagnosed cases of hypochloremic metabolic alkalosis have been brought to the attention of the Centers for Disease Control. One possible explanation for the delay in recognition of the problem is that, up to the present time, there has been very little information in the clinical literature with regard to simple dietary chloride deficiency. During the time when the extent of hypochloremic metabolic alkalosis among infants was being assessed, several medical hypotheses were proposed to try to explain the dietary and other conditions causally related to the development of hypochloremic metabolic alkalosis. As a result of 2 exploratory prospective studies using the dog as an animal model in one case and the miniature pig in the other, it is apparent that dietary chloride insufficiency alone does lead to hypochloremic metabolic alkalosis in normal animals comparable with that observed in infants.

The Infant Formula Act legislates a number of matters to ensure the nutritional safety of formulas by establishing specific requirements for nutrient content. Through regulation, the Secretary of the Department of Health and Human Services may revise this list of nutrients specified by law as new clinical knowledge is developed with respect to requirements in infant nutrition. The required level for any nutrient may also be revised, and requirements for quality factors for such nutrients may be established by regulation. Quality control procedures may be established by regulation to assure that an infant formula provides nutrients in accordance with the specifications of the Infant Formula Act. These quality control procedures include the periodic testing of infant formulas by the manufacturers to determine whether they are in compliance with the Act.

On December 30, 1980, FDA published a proposed rule for infant formula quality control procedures and allowed until March 2, 1981, for all interested persons to comment on this proposal. Three organizations requested additional time to prepare their responses, and the comment period was subsequently extended to May 1, 1981. FDA received comments from all American infant formula manufacturers, the principal trade association, the American Medical Association, the American Academy of Pediatrics, a consumer organization (Formula), and over 100 individuals. Consumers either supported or called for more stringent requirements. On the other hand, industry and the professional organizations found the regulations inflexible and potentially too costly. FDA's Bureau of Foods has evaluated all comments and is now in the process of drafting a Federal Register publication, which FDA hopes to publish within the next few months. This document will be more flexible than the proposal while at the same time continuing to ensure the nutritional quality of infant formula products. (The final rule on infant formula quality control procedures was published on April 20, 1982, in the *Federal Register* **47** (No. 76), pp. 17016–17027.)

Drawing from recommendations made by the Committee on Nutrition of the American Academy of Pediatrics in 1976, and subsequently reaffirmed in 1980, the Act establishes minimum and, in a few cases, maximum levels for protein, fat, essential fatty acids, and 26 vitamins and minerals. The only difference between the recommendations of 1976 and 1980 is that the minimum level for vitamin E was increased from 0.3 to 0.7 international units. All nutrients are required to be present at these levels per 100

kilocalories. Possibly because of oversight when the Infant Formula Act was written, nothing is stipulated about a minimum energy density of an infant formula as it is normally prepared in ready-to-feed form. The Committee on Nutrition of the American Academy of Pediatrics did specify an energy level of 670 kcal/L of formula. The Act specifies that, at the minimum level, the source of protein shall be at least nutritionally equivalent to casein. The minimum and maximum values for vitamin A were given as retinol equivalents. The Act also specifies a minimum and a maximum value for the ratio of calcium content to phosphorus content.

The minimum level for iron content in infant formulas is appreciably below that contained in the current regulation for infant foods (21 CFR 105.65) as well as the 1980 Recommended Dietary Allowance (RDA) established by the Food and Nutrition Board of the National Research Council. For practically all intents and purposes, the Infant Formula Act supersedes the current regulation on infant foods. The latter requires, for example, that a formula which contains less than 1 mg iron/100 kcal bear a label statement to the effect that an additional quantity of iron should be supplied from other sources. In their 1976 statement, the Committee on Nutrition of the American Academy of Pediatrics recommended that infant formulas contain an amount of iron equal to the lower end of the range commonly found in human milk, a value of about 0.15 mg iron/100 kcal, and that the iron be in a bioavailable form. This is the level that was incorporated into the Infant Formula Act. Part of the rationale for maintaining the minimum level is to permit some flexibility in the selection of foods and formulas for those infants who may be sensitive to some forms of iron. The Committee on Nutrition also affirmed its recommendation that infants at risk for iron deficiency be given formulas supplemented with iron at levels between 1 and 2 mg/100 kcal. Earlier in 1981, the Committee on Nutrition of the American Academy of Pediatrics maintained its position that the minimum level for iron is 0.15 mg/100 kcal, and also indicated that it believes that the caution statement for infant formulas containing less than 1 mg/100 kcal is appropriate. The Food and Drug Administration agrees with these recommendations and, in a letter to the Infant Formula Council, indicated that the provision of 21 CFR 105.65 for a label statement for formulas containing less than 1 mg iron/100 kcal is still appropriate and should continue to be provided by infant formula manufacturers and distributors.

These very brief comments on iron address only part of a much larger issue. From a regulatory standpoint, one critical aspect centers on what level of iron is considered to be appropriate to support claims that a product is fortified with iron. A corollary deals with the achievement of some uniform labeling practice for all infant foods. Issues such as these are expected to be part of the proposal for revision of 21 CFR 105.65, or a new proposed rule, which should be published in the near future. As is the normal course of events, FDA will seek comments on the proposal in order to develop the best possible regulation.

The list of nutrients in the Infant Formula Act is not necessarily all inclusive. In their 1980 edition of "Recommended Dietary Allowances," the Food and Nutrition Board of the National Research Council established what is termed an "estimated safe and adequate daily dietary intake" for 4 trace elements that are not listed in the Act, namely, fluoride, chromium, molybdenum, and selenium. In contrast to a recommended allowance, which is a single value, the estimated safe and adequate daily dietary intakes are presented as ranges of intake because it is felt that the available data do not permit the definition of a single intake for these nutrients. The Secretary of the Department of Health and Human Services can, through regulation, revise the list of nutrients if such an action is considered appropriate.

Infant Formula Physical Stability

J. PRESTON MILES[1]
Infant Formula Council, Atlanta, GA 30342

The physical nature of liquid infant formula products is characterized in terms of emulsion, suspension, and solution processes. Such mixtures inevitably undergo phase separation. A variety of physical and chemical phenomena govern the gradual changes in the appearance of these products. The consequent loss of physical elegance is primarily a question of consumer acceptance. Only in the most extreme cases do the physical characteristics deteriorate to the point that swallowing and digestion are affected. Recently, both public and regulatory attention has been focused on the physical characteristics of liquid infant formula products. Formula appearance is the product aspect most readily evaluated by the consumer. The design and manufacture of products that will have consistent appearance over extended periods is a difficult technical problem. This presentation summarizes the phenomena responsible for changes in the physical characteristics of liquid infant formula products. Product defects are discussed and methods for product evaluation are described. Representative data on the effect of storage on product physical elegance also are presented.

Commercial liquid infant formulas are essentially suspensions of protein, microemulsified oils, and sparingly soluble minerals in solutions of carbohydrates and minor ingredients. The nature of these mixtures inevitably leads to physical separation much as in the case of evaporated milk. Modern processing technology allows the production of infant formula in which the process of physical separation is retarded.

A wide variety of infant formulas currently are available. Infant formula products are produced principally in 3 forms: a ready-to-use liquid; liquid concentrate, which is diluted before use; and powder, which is designed to be reconstituted before use. Special products with caloric levels and formulations designed to satisfy various feeding requirements come in one or more of these 3 forms. Because each product contains a unique combination of ingredients and is processed by specialized means, the physical and chemical characteristics of each are slightly different. To describe each of these peculiarities in detail would be unnecessarily complex. Also, because the powder form does not display readily apparent changes in its physical nature, this discussion concerns chiefly the factors governing liquid infant formula products. Because of space limitations, only those changes are described that can potentially affect the product homogeneity or feeding characteristics or in some way influence the consumer's perception of overall product quality.

A brief summary of the materials used in infant formula is presented as background to a somewhat more detailed discussion of the physical and chemical phenomena that govern the mixtures. The visual defects that arise out of the progressive changes in product appearance are described, and several methods for evaluation of these defects are presented.

Composition of Products

Table 1 presents a summary of the ingredients commonly encountered in infant formula products.

The protein source for the majority of infant formulas is either cow's milk protein or soy protein. Only the butterfat-free forms of cow's milk are used, i.e., nonfat dry milk and condensed skim milk. Soy proteins in the form of soy isolates and as soy flours are used as raw materials. In some products, sodium and calcium caseinates and partially demineralized whey proteins are also used. The selection of these proteins is based primarily on nutritional considerations. The unique chemical composition of these protein types accounts for many of the differences in the physical behavior of products derived from them.

The lipid requirements of the infant are supplied by various refined vegetable oils, principally soy, coconut, and corn. Safflower, oleo, and medium chain triglyceride oils are used in other formulas. The choice of oils is based on the degree of unsaturation and balance of individual fatty acids as dictated by nutritional considerations.

Lactose, sucrose, and corn syrup are the primary carbohydrate sources. When corn syrups are used, the average chain length of the carbohydrate polymers is important from both a nutritional and physical (primarily viscosity) standpoint.

Presented at the Symposium on Infant Formula Regulation and Infant Food Problems, 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

[1] Present address: Centre College, Danville, KY 40422.

0004-5756/82/6506-1432-06$01.00

Table 1. Representative ingredients of infant formulas

Protein sources	nonfat dry milk, condensed skim milk, partially demineralized whey, Na and Ca caseinates, soy protein isolate, soy milk
Oils	soy, coconut, corn, safflower, MCT (medium chain triglycerides)
Carbohydrates	lactose, sucrose, corn syrup, and corn syrup solids
Minerals, major	calcium carbonate, mono-, di-, and tribasic calcium phosphates, dibasic magnesium phosphate, potassium citrate, magnesium chloride
minor	potassium iodide, ferrous sulfate, manganous sulfate cupric sulfate, zinc sulfate
Vitamins	A, E, D, K, C, B_1, B_2, B_6, B_{12}, niacin, folic acid, pantothenic acid, biotin, choline, inositol
Functional ingredients	soy lecithin, mono- and diglycerides, starch, carrageenans

A variety of soluble and sparingly soluble minerals are included in the products to meet the rigorous nutritional needs of the growing infant. The amounts, forms, and ratios of the major mineral ingredients (i.e., calcium carbonate, calcium phosphate, potassium citrate, magnesium chloride, and magnesium phosphate) have a major influence on the behavior of the protein system. Stabilizers and emulsifiers are used to retard the separation of these formulas over time. Trace elements and oil- and water-soluble vitamins are included to assure nutritional adequacy. These minor nutrients have little effect on the ultimate physical stability of the formulas.

Protein System.—Proteins are large macromolecules exhibiting a variety of chemical and physical properties. The behavior of this diverse class of materials dominates the stability of infant formulas more than any other component.

The physical behavior of a protein is governed primarily by the number and relative strengths of ionizable groups present on that protein. For any protein type, there exists a pH, called the isoelectric point, at which the number of positively and negatively charged groups is exactly equal, resulting in the protein having no net charge. This important property is a characteristic of that protein and is the pH at which minimum solubility is observed. At pH values greater than the isoelectric point the presence of certain cationic salts, primarily the divalent cations of calcium and magnesium, can reduce protein solubility.

The effect of heat on protein is to disrupt the salt linkages and hydrogen bonds that maintain the coiled or folded arrangement of the polypeptide in a stable globular form. Once disrupted, the protein relaxes into a more random configuration, exposing a higher proportion of ionizable groups. In such forms, the proteins are less soluble and more reactive. The parameters that most strongly affect the stability of the suspension of the protein component in infant formula are then pH, concentration of divalent cations, and degree of heat stress.

The exact manner in which a protein responds to changes in these factors depends, of course, on the nature of the protein itself. At least a dozen proteins have been identified in cow's milk. The physical and chemical behavior of many of these protein types is similar, and for our purposes we need consider only 2 general classifications: casein and whey proteins. A more detailed discussion is provided by Gordon and Kalen (1). Casein represents roughly 80% of the total cow's milk protein; the whey fraction accounts for the remainder. The casein fraction in milk has been shown to exist as complex particles, or micelles, also containing calcium, inorganic phosphate, magnesium, and citrate. These calcium caseinate-phosphate micelles range in size from 40 to 250 nm. The casein micelles are sensitive to changes in their ionic environment. Even at pH values well above the isoelectric point, the casein micelles agglomerate readily in the presence of calcium and magnesium. These aggregations eventually become large enough to precipitate. This is one of the major mechanisms for separation in infant formula. The whey proteins by comparison are less sensitive to salt effects but are more readily denatured by heat.

Soy proteins are globular in nature and exhibit isoelectric points between 4 and 5. Several distinct classes have been identified (2); the major fractions are denoted 2S, 7S, and 11S. (S is the Svedberg unit, a measure of the sedimentation rate under ultracentrifugation and thus an approximation of molecular weight.) The soy proteins are also sensitive to salt concentration and are denatured by heat.

The Lipid System.—The lipid fraction of infant formulas is present as a stable emulsion of microscopic spherical globules of vegetable oils dispersed in the aqueous medium. The size of globules varies somewhat but the majority have diameters less than 1 μm. Like other stable oil-in-water emulsions, a third material, the emulsifying agent, is required at the interface. Monoglycerides and soy lecithin serve this role and reduce the interfacial tension between the

2 phases. These chemicals form a monolayer at the interface with their more polar sections oriented to the aqueous phase. The proteins present in infant formula also contribute in a major way to the emulsification process. A mechanism similar to that observed in cow's milk is possible where distinct monolayers of protein encircle the fat globule.

Minerals and Stabilizers.—Mixtures of sparingly soluble and essentially insoluble salts are often employed to ensure adequate levels of calcium and phosphorus. In products based on cow's milk, significant amounts of calcium and phosphorus exist in colloidal un-ionized forms. Soy protein contains relatively less of these elements, and fortification is necessary. The development of stable suspensions of calcium phosphate is difficult and some degree of sedimentation is almost invariably encountered with extended storage. The rate of sedimentation is governed by particle size and the viscosity of the product. The sediment layer formed is generally not dense and is readily resuspended with vigorous agitation.

Stabilizers are used to increase product viscosity; carrageenan and starch are the most commonly used materials for this purpose. Carrageenan is a sulfonated galactan extracted from red seaweed; it interacts with protein to form a weak gel structure that serves to retard separation of lipid globules or insoluble mineral particles. Carrageenan has also been shown to function by interacting with the casein micelle and producing forms that are more stable with respect to precipitation by calcium ions (3).

Physical Stability Factors

Fluid infant formulas are complex systems in which gradual changes of a chemical and physical nature over time are inevitable. These changes include creaming, protein agglomeration, excessive gelation, and sediment formation. These defects are discussed in turn, and their progressive stages, reversibility, and severity are detailed.

Creaming.—The formation of a cream layer on whole milk is not commonly seen today because mechanical homogenization of commercial fresh milk is almost universal. Homogenization, however, is not always sufficient to prevent creaming over the lengthy storage periods common for infant formulas. It is important to distinguish between the phenomenon of creaming and that of actual fat separation.

In formula products, creaming is the natural development during quiescent storage of an upper layer enriched in fat globules. The globules themselves remain distinct and the cream can be redispersed with brisk agitation. Creaming begins almost immediately after processing and continues throughout the storage period. Occasionally, the cream layer coalesces and is difficult to disrupt without extraordinary means. Actual fat separation, also called "oiling off," occurs when the fat emulsion breaks down or is never completely established initially. Instances of fat separation are easily recognized by the clear to yellowish oil droplets or even oil layer found on the top of the product. This free oil cannot be re-incorporated even with the most vigorous agitation. Separation of the oil into a distinct phase (oiling off) is a rare phenomenon often related to adverse storage conditions.

Protein Agglomeration.—As discussed, proteins are denatured by heat and the action of polyvalent cations. Both elements are certainly present in the processing of infant formulas. One manifestation of partial denaturation can be the formation of visible agglomerations of protein. Protein micelle formation is a natural phenomenon in cow's milk, and similar calcium protein phosphate micelles occur in both soy and cow's milk formulas. These micelles are in the 40–200 nm size range and are certainly too small to be seen by the unaided eye. Under the influence of the heat of processing and in the presence of calcium and magnesium cations, these micelles tend to agglomerate into particles in visible size ranges. Such action is well known in the evaporated milk industry where the term "grain" is used to describe the particles. The mechanism of formation is probably electrostatic, because changes in product pH and calcium and citrate levels influence particle formation. The product defect involved is then the formation of macroscopic protein clusters. These particles incorporate calcium phosphate and may entrap fat globules. In extreme cases, the particles become large and numerous enough to clog the nipple during feeding.

It would be wrong to conclude that protein agglomeration is necessarily always a negative process. Some slight denaturation is in fact intentional because this increases the viscosity of the mixture. Slightly elevated viscosity is advantageous in reducing the rate of creaming and mineral sedimentation and yields a product with better "body" from a consumer viewpoint.

Gelation.—A third defect, in many respects related to protein agglomeration, is the formation of a somewhat rigid gel structure. Polysaccharide stabilizers interact with protein to

develop a loose gel structure that produces a more stable product. Both the protein sources and the stabilizers are natural products that vary in their reactivity. This fact, amplified by the effect of heat during processing, can sometimes lead to more extensive gel formation than desired.

Along with the above reactions, and no doubt related to them, is the process of eventual viscosity increase with extended storage. The phenomenon of age thickening is encountered in high temperature and short-time processed sterilized evaporated milk. Age thickening is observed after several (5–8) months of storage. Gel development is preceded by a rapid rise in product viscosity. The unique feature is that viscosity does not increase gradually but does so abruptly after some induction period. In rare instances, gelation may proceed to the extent that syneresis or "wheying off" occurs. In such cases, a somewhat clear "serum" zone will be formed in the bottom of the container due to the aggregation of proteins and entrapment of lipids in the upper gel layer.

The gelation encountered in aged infant formula products is not at all firm. Even mild agitation will disrupt the gel and restore the product to a uniform appearance. With such an easily reversible condition, one would hardly consider gelation a serious problem. The negative aspect is that, in the eyes of the consumer, the lumpy appearance of the formula upon initial stirring or merely uneven pouring characteristics are reminiscent of the appearance of bacteriologically spoiled milk. From that standpoint, gelation is perceived as a serious defect.

This similarity in appearance between gelation and the denaturing action of the lactic acid by-product of microbiological activity is often a source of confusion. The confusion is unfortunate, because it may cause needless concern and economic loss on the part of the consumer. Microbiological spoilage can generally be differentiated from gelation by the offensive odor and low pH (<6.0) produced by the former. A clear appreciation of the difference between these defects would be beneficial to consumers, regulatory agencies, and producers alike.

Sedimentation.—Sedimentation of inorganic salts occurs in infant formulas that are heavily fortified with insoluble minerals. Mineral fallout is encountered primarily in products based on soy protein and demineralized whey. In some cases, particles of denatured protein also form a sediment. The viscosity of the products, use of stabilizers, and small particle size of the insoluble salts retard the formation of the sediment.

Physical Stability Evaluation

Many procedures have been suggested and applied with success in particular situations. In an attempt to develop more general procedures, the Infant Formula Council has described a set of tests for the evaluation of these products. These procedures are intended to be straightforward enough so that skilled observers can make meaningful evaluations even though they are not familiar with the products. In addition, it is hoped, through the distribution and eventual application of the procedures, that regulatory officials, public health officials, and nutritionists can become more familiar with the products. A copy of the procedures is available from the Infant Formula Council.[2]

These tests do not cover all possible product defects but do allow examination of the most common problems encountered by the consumer. The tests are more sensitive than the normal observations made by consumers, thereby allowing detection of changes before the physical stability becomes unacceptable.

Creaming.—Creaming is evaluated by agitating the container well, then pouring the entire contents of the container into a clean, dry beaker and back again. Agitation serves to re-incorporate any slight cream accumulations and allows evaluation of cream particles which are too firm to re-incorporate. No cream (or only very small streaks on the surface) is the best rating. When the entire surface is covered with cream particles, the rating is considered poor. Such a product, although still nutritionally satisfactory, is generally not aesthetically acceptable to the consumer and is below the quality standards which the industry attempts to maintain. It must be re-emphasized that this is an evaluation of creaming and not of fat separation; de-emulsification leading to separation of the oil is most unusual.

Protein Agglomeration.—The determination of protein agglomeration is probably the most difficult evaluation to make. The container is shaken well and the contents are poured into a clean dry beaker and back again. The residual film of formula on the side of the beaker is observed. The size and number of agglomerated protein particles are evaluated. A completely uniform particle-free film or one with only a few

[2] Infant Formula Council, 5775 Peachtree-Dunwoody Rd, Suite 500-D, Atlanta, GA 30342.

Table 2. Physical stability of infant formulas; extended storage study[a]

Stability factor	Period, mo.	Excellent, %	Good, %	Poor, %	No. of lots
Gelation	3	100	0.0	0.0	168
	6	100	0.0	0.0	182
	12	100	0.0	0.0	42
	18	83	17.0	0.0	184
Creaming	3	98	1.2	0.6	168
	6	100	0.0	0.0	182
	12	65	35.0	0.0	42
	18	58	41.0	1.0	184
Protein agglomeration	3	99	1.2	0.0	168
	6	99	1.0	0.0	182
	12	100	0.0	0.0	42
	18	58	40.0	2.0	184

[a] Ready-to-use milk-based formula, room temperature storage. Shelf life stated as 18 months.

small protein particles is rated best. When protein agglomeration particles are readily visible, irregular in shape, and roughly as large as fine sand particles, the rating is considered poor. Product samples with this degree of protein agglomeration may occasionally clog nipples during feeding and for that reason are unsatisfactory. Some caution is needed in making these evaluations. The inexperienced observer often confuses small flecks of cream with the protein particles. The cream flecks appear 3-dimensional in the liquid film and are much less numerous than the protein particles.

Gelation.—Evaluation of gelation must be performed on an unagitated container of formula. The container is opened, the contents are then poured slowly out of the container, and the appearance of the stream of product is observed as it is being poured. On the basis of these observations, the gelation is rated. If the fluid surface is completely smooth as it flows over the lip of the container, it is considered to be free of gelation. If there is very soft gel structure which on pouring disperses easily, the product is considered acceptable. If large gel-like masses "plop" from the container on pouring and remain throughout the product after pouring, the product is rated poor.

Effects of Storage

The chemical and physical processes observed in infant formulas and the product defects which may result are all time-dependent phenomena. The reactions governing these changes are all slow at normal storage temperatures. However, on a time scale of several months, changes in the physical characteristics can be observed.

Infant formula manufacturers are familiar with the rate at which physical defects develop through extended storage studies and previous experience. Therefore, they have established product shelf life accordingly. The gradual loss of physical elegance is, in almost all situations, the limiting condition in establishing product life recommendations.

An example of a study of the effect of storage on an infant formula is given in Table 2 for a ready-to-use, milk-based product. In this experiment, changes in creaming and protein agglomeration begin to occur at 12 and 18 months, respectively. By no means are these product parameters always the first to deteriorate beyond acceptable levels. Because of the wide variety of ingredients and processing systems used, generalizations on critical parameters or predictions of appropriate shelf life are almost impossible to make.

Summary

The processes of solution, emulsion, and suspension were shown to describe the physical stability of liquid infant formula. The major ingredients and the nature of those ingredients were described in terms of the product defects which may be observable to the consumer. Methods for the evaluation of physical stability were described. Limited data were presented that indicate that the rate of development of the defects is slow. For the particular product studied, less than 5% of the lots tested exhibited, at the end of shelf life, a degree of physical stability change which would be apparent under normal use conditions. In view of the fact that the changes are largely ones of consumer acceptance and not changes in nutritional ade-

quacy, confidence can be expressed in the quality of products available to the consumer.

REFERENCES

(1) Gordon, W. G., & Kalen, E. G. (1974) in *Fundamentals of Dairy Chemistry*, 2nd Ed., B. H. Webb, A. H. Johnson, & J. A. Alford (Eds), AVI Publishing Co., Westport, CT

(2) Wolf, W. T. (1978) in *Soybeans: Chemistry and Technology*, A. K. Smith & S. T. Circle (Eds), AVI Publishing Cc., Westport, CT

(3) Lin, C. F., & Hansen, P. M. T. (1970) *Macromolecules* **3**, 269

Proximate and Elemental Analysis of Infant Formula

JAMES T. TANNER
Food and Drug Administration, Division of Nutrition, Washington, DC 20204

The Nutrient Surveillance Branch has been conducting a survey of infant formula products for Fiscal Year 1981. Each product has been carefully analyzed and the results compared to the label declaration and the minimum-maximum limits specified by the American Academy of Pediatrics' Committee on Nutrition (CON/AAP). Proximate and elemental analyses were made. Protein, fat, ash, and total solids (moisture) were determined by AOAC methods. Osmolality, density, and fatty acids (linoleic) were also determined. Carbohydrates were calculated by difference and caloric content was calculated by using the general Atwater factors. Elemental analysis for Ca, P, Mg, Fe, Zn, Cu, Mn, Na, and K were performed by induction coupled plasma absorption spectroscopy. Chloride was assayed by potentiometric titration with $AgNO_3$. A summary of the findings from the infant formula survey have been compared with CON/AAP recommendations. In general, there were only a few exceptions where the label claims and the CON/AAP requirements were not met. However, in none of these cases was the difference considered to be of public health significance.

During 1979, the Food and Drug Administration (FDA) learned of an infant formula being produced which was low in chloride content and which caused hypochloremic metabolic alkalosis in infants when used as the primary source of nutrition. Although only one manufacturer had been identified as producing a deficient formula, there was growing concern by the public about the quality of the other infant formulas on the market. The FDA initiated a surveillance program in which samples of infant formulas were collected from all of the manufacturers so that the nutrient content could be assayed.

The nutrient content of the infant formulas was assayed and compared with the label declaration and with the minimum requirements for nutrients given by the Committee on Nutrition of the American Academy of Pediatrics (CON/AAP) in 1976. These requirements were revised in 1980. The FDA's infant formula survey program is still underway and is expected to continue for some time in order to fulfill the requirements of the Infant Formula Act of 1980.

This paper reports on the results of that survey through Fiscal Year (FY) 1981 for proximate and elemental content.

Elemental Content

The Infant Formula Act of 1980 lists minimum levels for the elements calcium, phosphorus, magnesium, iron, iodine, zinc, copper, manganese, sodium, potassium, and chloride and maximum levels for sodium, potassium, and chloride. With the exception of iodine and chloride, all elemental analyses were performed by inductively coupled plasma optical emission spectroscopy (ICP).

ICP is a technique based on the excitation of atoms in a hot discharge. The emission of ultraviolet and visible light from the atoms, excited in the discharge, is then monitored. The concentration of a particular element is determined by comparing the emission intensity of the sample with the emission intensity of known standards containing the same element(s) of interest.

Before the ICP determinative step, samples were digested by a wet ashing procedure carried out as follows: A composite of each sample was made from 12 units of formula. An aliquot of the composite was transferred to a 100 mL Kjeldahl flask along with 30 mL HNO_3–$HClO_4$ (2 + 1) and a few glass boiling beads. The sample size used depended on the form of the sample (15 mL if the sample was ready-to-feed, 10 mL if the sample was concentrated, and 1.5 g if the sample was a powder). The samples were gently heated on a heating mantle until all reaction with perchloric acid had subsided. Digested samples were then diluted to 50 mL with distilled deionized water. Occasionally a slight precipitate would develop in some of the samples after digestion; this precipitate dissolved when the samples were allowed to sit overnight. The nature of the precipitate has not yet been investigated, although it has been suggested that it is a perchlorate salt, possibly of potassium.

Elemental determinations of the infant formulas were made by using a Jarrell-Ash Model 975 Plasma Atom Comp with automatic background correction. The system used in this work was capable of the simultaneous determination of 29 elements. Data handling was accomplished with the aid of a PDP-8 minicomputer.

Presented at the Symposium on Infant Formula Regulation and Infant Food Problems, 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

0004-5756/82/6506-1488-03$01.00

Forward power to the plasma was maintained at 1100 W with reflected power less than 5 W. The ICP was given a minimum warm-up time of 30 min. Observation height in the plasma was optimized on manganese, which was found to be 16 mm above the load coil.

A calibration curve for each element was constructed (by computer) from standards prepared in 20% perchloric acid to simulate the samples after dilution, thus eliminating variations in sample uptake and aerosol production by the nebulizer. Samples were then run against the calibration curve to determine elemental content.

A collaborative study is nearing completion for infant formula using the ICP. Results to date are very good and will be reported at a later time.

Chloride.—Chloride was determined by the AOAC potentiometric titration method for chloride in foods, **32.025-32.030.**[1] This method has been shown to be applicable to a wide variety of foods. Comparison of results by the potentiometric titration method with those by neutron activation analysis on the same infant formula samples show excellent agreement. In addition, analysis of National Bureau of Standards (NBS) Standard Reference Materials (SRM) further verified the method. It was important that the chloride determination be fully verified because a deficiency of this mineral was the basis for the metabolic alkalosis in the subject infants.

Chloride was determined in infant formula by diluting the sample, acidifying with nitric acid, and titrating the soluble chloride with standard silver nitrate to the potentiometric end point. The silver nitrate was standardized against NBS potassium chloride. In addition, several of the NBS SRMs were analyzed and the chloride values compared. In all cases, agreement was excellent.

Iodine.—Iodine was determined by a modification of the Technicon AutoAnalyzer II industrial method No. 530-77A. The samples were prepared for analysis by heating with alcoholic potassium hydroxide and ashing in a muffle furnace. The ash was dissolved in dilute sulfuric acid. The determination of iodide is based on the catalytic reduction of ceric ion. The color of the reaction mixture is proportional to iodide concentration.

Proximate Analyses

Protein.—The AOAC Kjeldahl method, **2.057,** was used to determine protein content. Basically, protein is converted to ammonium sulfate by digestion with sulfuric acid and catalysts. The ammonia is freed upon addition of base and distilled into 0.1N sulfuric acid, and the excess acid is titrated with 0.1N sodium hydroxide. The sodium hydroxide is standardized by titration with the primary standard potassium hydrogen phthalate and the acid is titrated against the sodium hydroxide. Nitrogen is converted to protein by using the factor 6.25. A blank is run with each set and includes everything except the sample, to determine contribution of any nitrogen from the reagents. Samples were analyzed in duplicate; the results agreed within 1-5%.

Density.—A known volume (10 mL) was weighed for the density determination. Each sample was determined in duplicate and the density was reported as the average of the 2 determinations. This number was used in the calculations of the other proximate analysis.

Moisture (Total Solids).—Moisture was determined by AOAC method **16.032.** Results were converted from percent by weight (w/w) to percent by volume (w/v) by multiplying by the density calculated above.

Ash (Minerals).—Ash was determined by using AOAC method **16.035.** Results were converted from percent by weight (w/w) to percent by volume (w/v) by multiplying by the calculated density.

Carbohydrate.—Carbohydrates were calculated by difference. The sum of the determined values for protein, fat, moisture, and ash expressed as percent (w/w) was subtracted from 100 to give carbohydrate in analogous terms. Grams of carbohydrates per serving were calculated by subtracting the value of the protein, fat, moisture, and ash from the declared serving size.

Calories.—Calories were also calculated on the basis of the other proximate results. The same values were used as in the carbohydrate calculation, including the calculated carbohydrate value. When available, specific Atwater factors for protein, carbohydrate, and fat should be used, as given in U.S. Department of Agriculture Handbook No. 74. When no specific factors are available, as in infant formula, the general values of 4 for protein, 4 for carbohydrate, and 9 for fat should be used. Calories are then calculated by the following equation when the proximate components are expressed as percent (w/w) or percent (w/v):

$$(4 \times \text{protein}) + (4 \times \text{carbohydrate}) + (9 \times \text{fat}) = \text{caloric content per 100 g or mL}$$

To obtain the value in fluid ounces, a ratio is used.

[1] All references to official AOAC methods are to *Official Methods of Analysis* (1980) 13th Ed. and its supplements.

(caloric content/100) = (x/30)
where x = calories per fluid ounce (30 mL).

Fat.—The fat content was determined as in AOAC method **16.172,** but with a smaller sample size (between 2 and 2.5 g). Hydrolysis of the fat depends on the type of infant formula: alkaline for soy-based samples and acid for milk-based formula.

Compliance with CON/AAP requirements as stated in the Infant Formula Act was determined by converting results to a 100 kcal basis by using determined caloric values per unit volume or unit weight.

Discussion

During 1979 when the low chloride infant formula was detected, an inspectional and sample collection program was initiated for sampling infant formula that accounted for about 95% of the total sales in the United States. Only commercial formulas intended for "normal" term infants were sampled; special formulations for infants of low birth weight, etc., were not sampled in the initial program. Two cases of formula were collected and shipped to FDA Headquarters laboratory for nutrient analysis. Each nutrient was compared with the label declaration and with the minimum/maximum values from the CON/AAP. Of the 29 formulas from 6 different manufacturers, only one formula was deficient with respect to the CON/AAP requirements. That formula also contained an insufficient chloride content; however, it was not as low as the earlier deficient formula from another manufacturer. An FDA Health Hazard Board review determined that no significant public health problem could be identified, because some samples of human milk had comparable levels of chloride. The company was notified and the product was reformulated.

At the conclusion of the first survey, the FDA initiated a program to sample infant formula on a much more rapid basis. For this program, samples were collected from the production line at the company rather than from the marketplace, and were shipped to the FDA for "immediate" analysis. "Immediate" in this case meant 2 weeks to determine the complete list of nutrients. During 1980–1981, approximately 75 infant formulas were analyzed and again compared with the label declaration and with the recommendation of the CON/AAP.

The results of the survey through FY 1981 for proximate and elemental analysis are shown in Table 1. When these nutrients are compared with the label claim, there is just a small overage, as would be expected. If all the nutrients shown here are averaged, the formula would contain 119% of the label claim. When compared with the CON/AAP requirements, the complete average is 451%, or 218% if iron and manganese are omitted. The high overage for iron is understandable because many formulas are fortified with iron at 1 mg/100 kcal in accordance with 21CFR 105.65. The high amount of manganese is not understood, and although it is not known to be harmful, the excess over the CON/AAP requirements does not seem to be justified.

Table 1. Results of analyses of infant formulas in the FY 1981 survey

Nutrient	Av. % of requirement	
	CON/AAP[a]	Label
Protein	151	100
Fat	163	101
Fatty acids	622	—
Calcium	178	106
Phosphorus	273	104
Magnesium	177	131
Iron	1133	137
Zinc	167	122
Copper	196	136
Manganese	2324	154
Sodium	200	113
Potassium	144	114
Chloride	131	108

[a] Committee on Nutrition of the American Academy of Pediatrics.

When the formulas are divided into milk-based and soy-based, the same general percentages appear for both. However, manganese is higher relative to the CON/AAP requirements in soy products than in milk products, namely, 3379% for soy and 1953% for milk.

In general, we may conclude that for proximate constituents and minerals the infant formulas currently being sold meet their label requirements and are well above the CON/AAP requirements.

Future Work

As a continuation of the work reported here, infant formulas for FY 1982 are still being analyzed at about the same rate as during 1981. This is expected to continue in order to comply with the Infant Formula Act of 1980 and to ensure that the infant formulas in the market place are safe and fully meet the nutrient standards.

Acknowledgment

The author thanks members of the Nutrient Surveillance Branch, FDA, who performed the analyses described in this paper.

Vitamin Analysis in Infant Formulas

ALVIN W. GRANADE[1]
Food and Drug Administration, 1182 W Peachtree St, NW, Atlanta, GA 30309

The methodology used by the Atlanta Regional Laboratory, U.S. Food and Drug Administration, for determination of vitamins in infant formulas is summarized. Analytical findings for 11 vitamins are presented by manufacturer in terms of the requirements of the Infant Formula Act and percentage of the manufacturer's label declaration.

The Infant Formula Act of 1980 (P.L. 96-359) was signed into law September 26, 1980. The Act established a nutrient content standard for infant formulas and specified the requirements that manufacturers must meet to ensure the safety and quality of their infant formula products. On November 20, 1980, the Food and Drug Administration notified the manufacturers that beginning December 26, 1980, they must submit regular reports confirming that their products contain all the nutrients essential for normal growth.

In November 1980, the Food and Drug Administration initiated an inspection program for infant formula manufacturers which included the collection and analysis of samples for nutrient composition. Each sample was analyzed for all nutrients as declared on the label or as required by the Act except vitamin D, vitamin K, choline, and inositol. This report summarizes the vitamin assay methodology used by the Atlanta Center for Nutrient Analysis and the assay results.

Five samples were analyzed from each of 5 manufacturers. The formulation types consisted of ready-to-use, concentrated liquids, and powders prepared in either a nonfat milk base or a soy base. All analyses were performed on the sample composite prepared by combining 12 units from the same lot. Liquid samples were mixed in a sanitized container under nitrogen. Powder samples were blended mechanically in a sanitized container.

The label instructions for the preparation of liquid samples presented no quantification problems. However, powdered formulations presented a problem in relating label instructions to declared levels. For example, to prepare a quart of formula from a powdered infant formula, the label indicated that one unpacked level cupful of powder was to be added to 29 fl. oz of water. Four samples (12 units per sample) examined from one firm had average powder weights per cup of 116.0, 116.9, 118.7, and 120.4 g, respectively. Individual powder weights per cup varied about ±5% from the average. A second laboratory found that a cup of powder weighed about 15% higher than our determined values. It would seem prudent to indicate quantifiable units (grams) in addition to household units in the label instructions.

Experimental

Vitamins A and E

All samples were assayed by a high pressure liquid chromatographic (HPLC) procedure developed in our laboratory. Each sample was homogenized in a Polytron mixer in a solvent mixture of 2-propanol and methylene chloride with magnesium sulfate added to remove water. After filtration, the extract was evaporated to dryness under vacuum and the residue was dissolved in methylene chloride, filtered, and evaporated to dryness under vacuum. The lipid residue was dissolved in methylene chloride and diluted to volume. A portion of this solution was subjected to gel permeation chromatography (3 μStyragel columns in series) to fractionate the vitamins from the lipid material. The vitamin fraction was collected and quantitated by nonaqueous reverse phase chromatography on a Zorbax ODS column. Vitamin A palmitate and vitamin E acetate were calculated from the UV response at 313 and 280 nm against standards similarly treated. The β-carotene content was measured at 436 nm.

For comparison, 6 samples were also assayed by AOAC method **43.008–43.013** for vitamin A.[2] This procedure involved alkaline hydrolysis, extraction of vitamin A in hexane, and Carr-Price colorimetry. Chromatographic cleanup was not required.

Vitamin C

All samples were assayed by AOAC method **43.061**. Each sample was extracted with *meta-*

Presented at the Symposium on Infant Formula Regulation and Infant Food Problems, 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

[1] Retired.

[2] All references to official AOAC methods are to *Official Methods of Analysis* (1980) 13th Ed. and its supplements.

0004-5756/82/6506-1491-04$01.00

phosphoric acid–acetic acid solution and the ascorbic acid was oxidized to dehydroascorbic acid by treatment with Norit. After filtration, a fluorophor was formed with *o*-phenylenediamine. Fluorescence was measured at 430 nm with activation at 350 nm. Blanks were determined by blocking fluorophor development with boric acid.

Vitamin B_1 (Thiamine)

Samples were subjected to acid and enzyme extraction as outlined in AOAC method **43.025–43.026**. The filtered and diluted extracts containing about 0.2 μg thiamine/mL were assayed by a benzenesulfonyl chloride (BSC) semiautomated procedure. Thiochrome fluorescence of sample extracts was measured before and after addition of benzenesulfonyl chloride and after the addition of thiamine standard. The BSC-treated sample was used to correct for blank fluorescence and an internal standard was used to correct for matrix quenching effects.

Vitamin B_2 (Riboflavin)

Two methods were used for riboflavin assay. Fifteen samples (Mfrs B, D, and E) were assayed by the AOAC microbiological method using *Lactobacillus casei* (ATCC No. 7469), **43.168** and **43.173–43.176**.

For the remaining 10 samples (Mfrs A and C), the vitamin B_1 extract was used to measure riboflavin by the semiautomated procedure described in AOAC method **43.B01–43.B04**, second supplement. The method was modified by the use of an internal standard rather than a standard curve and the determination of an enzyme blank.

Vitamin B_6

All samples were assayed by AOAC methods **43.191** and **43.193**. The column fractionation step specified for separation of the 3 forms of vitamin B_6 was not performed because all samples were fortified with pyridoxine hydrochloride. Where mixed standards were indicated by the method, only pyridoxine hydrochloride was used.

Vitamin B_{12}

All samples were assayed by the AOAC microbiological methods using *Lactobacillus leichmannii* (ATCC No. 7830), **43.134** and **43.139–43.141**.

Niacin

Twenty samples (Mfrs A, B, C, and E) were assayed by the AOAC automated chemical procedure, **43.047–43.051**.

Five samples (Mfr D) were assayed by the AOAC microbiological procedure using *Lactobacillus plantarum* (ATCC No. 8014), **43.150–43.158**.

Niacin Equivalents

Three manufacturers declared niacin levels in terms of niacin equivalents. This takes into account that, in humans, some dietary tryptophan is converted to niacin. For the purpose of establishing niacin equivalents, a dietary intake of 60 mg tryptophan is considered equivalent to 1 mg niacin. Therefore, these samples were assayed for tryptophan after alkaline hydrolysis using a Durham D-500 amino acid analyzer.

Folic Acid

All samples were assayed by the method of Ford et al. (*J. Dairy Res.* (1969) **36**, 435) as modified by the Division of Nutrition, Food and Drug Administration. The sample aliquots were diluted with ascorbic acid–phosphate buffer and digested with chicken pancreas extract overnight at 37°C. The enzyme was inactivated by autoclaving at 121°C for 5 min and the pH-adjusted extracts were assayed together with an enzyme blank prepared in a similar manner.

Total folates were determined microbiologically with *Lactobacillus casei* (ATCC No. 7469) per AOAC microbiological vitamin analysis protocols, **43.126–43.133**.

Free Pantothenic Acid

Each sample was assayed by a microbiological procedure compiled by the Division of Nutrition, Food and Drug Administration. The sample was extracted with Tris buffer and adjusted to pH 4.5. After precipitation of protein, pantothenic acid was determined microbiologically with *Lactobacillus plantarum* (ATCC No. 8014).

Biotin

Twenty samples were assayed by a microbiological procedure. A sample containing 20–80 μg biotin was hydrolyzed with 6N H_2SO_4 by autoclaving at 121°C for 1 h. After filtration, the solution was adjusted to a pH of 6.8 and diluted to a concentration of 0.1 ng biotin/mL. This solution was assayed by the AOAC microbiological turbidimetric methods using *Lactobacillus plantarum* (ATCC No. 8014), **43.126–43.133**.

Table 1. Average vitamin levels per 100 kcal in infant formula by manufacturer

Vitamin	Infant Formula Act requirements	Manufacturers				
		A	B	C	D	E
Vitamin A, IU	250–750	562	458	350	317	428
Range		500–625	437–493	337–380	269–359	301–606
Vitamin E, IU	≥0.7	2.60	1.31	2.01	2.11	1.94
Range		2.38–2.99	1.24–1.36	1.90–2.21	2.02–2.23	1.80–2.11
Vitamin C, mg	≥8.0	17.3	13.1	19.2	13.5	12.9
Range		16.1–19.3	10.7–15.8	12.7–25.1	11.7–15.4	11.5–13.8
Vitamin B_1, μg	≥40	163	147	104	125	94
Range		102–295	110–166	101–110	109–153	89–97
Vitamin B_2, μg	≥60	196	270	200	176	183
Range		124–275	189–305	143–241	138–289	144–210
Vitamin B_6, μg	≥35	129	105	117	99	64
Range		108–190	99–117	112–127	95–103	58–71
Vitamin B_{12}, μg	≥0.15	0.90	0.42	0.48	0.47	0.49
Range		0.55–1.15	0.39–0.49	0.40–0.54	0.38–0.56	0.45–0.56
Niacin, μg	≥250	1711	1131	1398	1023	1374
Range		1430–2275	905–1227	1310–1510	944–1059	1200–1560
Niacin equiv.		1879	NA[a]	NA	1353	NA
Range		1565–2476			1198–1595	
Folic acid, μg	≥4.0	16	11	37	17	18
Range		14–17	8.4–17	20–27	16–19	15–20
Pantothenic acid, μg	≥300	891	421	628	651	556
Range		714–1239	346–516	597–643	612–690	488–606
Biotin, μg	≥1.5	26.9	3.4	NA	11.7	1.28
Range		24–29	2.8–5.6		10.3–12.2	1.02–1.45

[a] No analysis.

Culture media used were Bacto-Lactobacilli broth, Bacto-Lactobacilli agar, and Bacto-Biotin assay medium.

Results and Discussion

The average levels and ranges of vitamins found per 100 kcal are reported by manufacturer in Table 1. All vitamin levels met the requirements of the Infant Formula Act. The manufacturer's average levels ranged from 140 to 684% of the required level except for biotin, which ranged to 1793%.

Nonmilk-based formulas are required to contain a minimum of 1.5 μg biotin per 100 kcal. Milk-based formulas are not included in this requirement. Although biotin was not declared on the label, 5 milk-based formulas from manufacturer E were assayed for biotin. The average level of 1.28 μg biotin per 100 kcal found was below the 1.5 μg level required for nonmilk-based formulas. A second laboratory found 4.5% higher results on one sample analyzed.

Two of 5 samples from manufacturer E had vitamin A levels approximately twice the level of the other 3. The variations did not appear to be related to formula type. Individual sample levels for manufacturers A, B, C, and D were within ±15% of their average level. Five samples from manufacturer B were also assayed for vitamin A by the AOAC Carr-Price method, **43.008–43.013**. The average of 520 IU/100 kcal found by this method was 13% (0–26%) higher than the HPLC procedure.

All manufacturers had variations from their average levels in excess of ±20% for some vitamins as follows: *Manufacturer A*—vitamin B_1 (63–181), vitamin B_2 (63–140), vitamin B_6 (84–147), vitamin B_{12} (61–128), niacin (84–133), and pantothenic acid (80–139); *Manufacturer B*—vitamin B_1 (75–113), vitamin B_2 (70–113), folic acid (76–154), biotin (82–165), and pantothenic acid (82–123); *Manufacturer C*—vitamin C (66–131), vitamin B_2 (71–120), and folic acid (64–119); *Manufacturer D*—vitamin B_1 (87–122) and vitamin B_2 (78–164); *Manufacturer E*—vitamin A (70–144) and vitamin B_2 (79–115).

The averages of percent of labeled declarations found are reported by manufacturer in Table 2, together with the range of findings. Each manufacturer's average percent of declared found for all vitamins exceeded the declared level except vitamin E (93.4%) for manufacturer B. For some manufacturers, individual sample results were less than 100% of declared for vitamins A, E, B_6, niacin, and folic acid; the lowest result was 86% (vitamin A). Forty-five of 52 of the average results were between 93 and 200% of the label declaration. Vitamin B_{12} showed the

Table 2. Average percent of labeled vitamins found in infant formula by manufacturer

Vitamin, % of decl.	Manufacturers				
	A	B	C	D	E
Vitamin A	147	117	144	101	171
Range	135–159	112–126	135–152	86–115	120–242
Vitamin E	107	93.4	107	233	103
Range	94.7–135	88.0–97.7	102–118	95–272	95–112
Vitamin C	208	152	236	144	160
Range	197–237	124–184	156–308	125–165	142–170
Vitamin B_1	177	141	133	160	120
Range	162–213	105–159	130–141	139–196	114–124
Vitamin B_2	153	173	213	188	195
Range	114–186	121–195	153–257	147–308	154–224
Vitamin B_6	190	169	186	159	103
Range	171–204	158–188	180–204	152–164	92–114
Vitamin B_{12}	302	235	152	150	156
Range	127–520	155–264	129–174	122–175	144–178
Niacin	ND[a]	ND	111	ND	110
Range			104–120		96–125
Niacin equiv.	145	NA[b]	ND	110	ND
Range	127–159			105–128	
Folic acid	105	142	196	110	111
Range	87–118	107–218	126–235	101–119	94–125
Pantothenic acid	147	123	134	139	118
Range	119–168	110–146	127–137	131–147	104–129
Biotin,	122	127	ND	150	ND
Range	112–132	101–136		132–160	

[a] Not declared.
[b] No analysis.

highest average (302%) and highest individual value (520%).

The individual sample variations from their respective manufacturer's average percent of declared for vitamin B_6, niacin, and pantothenic acid were within twice the estimated maximum analytical error (±10%). The remaining vitamins showed variations by at least one manufacturer in excess of twice the estimated maximum analytical error as follows: *Manufacturer A*—vitamin E (88–126), vitamin B_2 (74–122), and vitamin B_{12} (42–172); *Manufacturer B*—vitamin B_1 (74–113), vitamin B_2, (70–113), vitamin B_{12} (66–112), and folic acid (75–154); *Manufacturer C*—vitamin C (66–131), vitamin B_2 (72–121), and folic acid (64–120); *Manufacturer D*—vitamin E (41–117), vitamin B_1 (87–122), and vitamin B_2 (76–164); and *Manufacturer E*—vitamin A (70–141).

Nutrient Overages and Methodology Trends

DAVID L. MAYS
Mead Johnson & Co., Nutritional Quality Control, 2400 Pennsylvania Ave., Evansville, IN 47712

Because infant formula provides the sole dietary intake for many infants, it is important that the nutrient composition be carefully controlled. Establishing nutrient levels depends on laboratory testing. Current testing methods have certain limitations, some of which can be overcome by modern trends in analytical technology.

Factors Affecting Composition

Minimal nutrient needs, and in some cases maximal tolerances, for normal infants have been developed by the Committee on Nutrition of the American Academy of Pediatrics (AAP/CON). These have been translated into guidelines for infant formula and formalized as part of the Infant Formula Act of 1980. The infant formula industry designs products within these ranges and publishes, on each container of formula, the label claim amount of various nutrients. The manufacturing industry aims for each product to provide at least the amount of nutrient claimed on the label of the product, throughout the shelf life of the product. This means that any time a customer buys a product, it should have all the nutrients listed on the label and at least the amount shown. How does a company make sure that this actually happens?

For a variety of reasons, the amount of a nutrient initially put into a product may not remain constant throughout the lifetime of the product. Some of these factors include the following:

At the time of manufacture: composition of raw materials; processing losses.

During shelf life: specific product matrix effects; product form; product packaging; product use. It may be useful to look separately at variables which affect nutrient levels at the time of manufacture and variables which cause losses during the shelf life.

Infant formulas are made primarily from natural materials, such as skim milk, plus other added nutrients. Natural materials contain many nutrients whose levels tend to vary somewhat as a function of geographical source, time of year, etc. As an example, the levels of several nutrients in milk vary as cows change from pasture to dry feeds for their primary source of nourishment. For some nutrients the levels present, such as the iodine content of milk, may periodically exceed the label claim of the formula, resulting in a naturally occurring overage. For most vitamins only a minor portion of the nutrient is provided by the natural ingredients; the bulk of the nutrient content is added separately. Vitamins added to formula come from synthetic or semisynthetic processes. These nutrients are more highly controlled; their composition is known, and they are assayed before use.

The manufacturing process also affects the amounts of nutrients in the final product. Certain vitamins are heat-labile and are affected to various degrees by the equipment and conditions used for processing and sterilizing. Losses may depend on the number of steps and the types of processes to which the nutrients are subjected. For example, if vitamins are added at the beginning of a multi-step process, losses due to thermodegradation or oxidation may be greater than if they are added near the end of the processing.

A number of factors also affect the nutrient composition from the time a product is manufactured until it is consumed. Each product formulation is a unique matrix in which any number of possible interactions may occur over time. Some of these interactions can be predicted from previous experience or inhibited by appropriate formulating techniques. In general, for example, there are fewer chemical interactions and vitamins are more stable in powder products than in liquid products. Furthermore, liquid products with different caloric densities may show different stability patterns. However, these generalizations serve only for guidance; long experience has shown that the effects of age on vitamin content of any given product can be reliably assured only by appropriate laboratory analysis.

Product packaging can have an effect on nutrient composition from more than one aspect. Possible interactions of the product with the package must be considered. These are generally undesirable and must be avoided by design.

Presented at the Symposium on Infant Formula Regulations and Infant Food Problems, 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

0004-5756/82/6506-1495-05$01.00

Clear containers which allow exposure of product to light will result in some loss of vitamins sensitive to light. The actual nutrient content at the time of consumption may be affected by the actual method of use. For example, reconstitution of formula powders requires mixing or shaking with water, which introduces some air into the product. Some oxidative losses of vitamin C must be expected and consequently compensated by proper formulation. These are just a few examples of processing and shelf life losses of nutrients.

Considerations for Establishing Overages

Because the nutrient content of a product may change during processing and over its shelf life, the manufacturer adds overages of some nutrients to compensate for the changes. How does the manufacturing company determine the amount of each nutrient to add to a product? In the case of existing products, manufacturers continually test samples at the time of manufacture and after prolonged stability periods to monitor the nutrient composition of each product and to determine that products continue to meet label claims during the shelf life.

For the introduction of new products, a great deal of study must go into the design of the formulation. Frequently this will include considering the composition of similar products, thoroughly studying the nutrient levels in anticipated raw materials, and providing overages in consideration of the factors we have already discussed. The industry generally formulates on the conservative side, so that in calculating the nutrient contributions from raw materials the lowest expected level will be used. That is, if seasonal or geographical variations are substantial, the lowest anticipated level of nutrient will be used for calculating how much additional nutrient to add to the product. Furthermore, enough overages will be added to compensate for processing and storage losses. Overages must be designed for each nutrient for each individual product. Prototype batches are made, first in the laboratory and then in the plant, and the batches are put on test. Products are evaluated over time by testing, and formulations are modified as indicated by the test results.

It should be remembered that the aim is to formulate and manufacture a product which will meet the label claims throughout the product shelf life. Such overages as are shown necessary by product testing are built into each product. If uncertainties exist, it is usually better to err toward overages, but not excessively so.

Method Limitations

It is obvious from the above discussion that formulating decisions are based on the results of laboratory testing. The accuracy of such decisions depends on the adequacy of the analytical data. It would probably be worthwhile to consider analytical testing and attempt to identify limitations or shortcomings in testing methods. Some factors which affect the usefulness of analytical results include the general type of method, i.e., biological versus chemical or microbiological test methods; method specificity and accuracy; additional information from new methods; and availability of reference materials.

Products are formulated to contain certain amounts of vitamins in order to obtain particular biological effects. Research has shown that vitamin activity of a particular type may be provided by more than one chemical compound. For example, vitamin A activity may be obtained from a number of compounds of the carotene family, as well as from several isomers and esters of retinol. These different chemical forms differ in biological activity, either on a weight or on a molecular basis. Furthermore, the amount of vitamin activity from a particular compound may depend to some degree on the species of animal and to a lesser degree on the physiological state of the animal. Therefore, the amount of vitamin or vitamin activity measured in a natural material depends on the conditions and assumptions made for measurement.

In the past, most vitamin determinations were measurements of biological effects on animals. In this way, the biological activity of materials of ill defined composition could be measured. The official methods for vitamins D and K are still biological methods. Otherwise, animal methods have been abandoned where suitable chemical and microbiological methods have become available. Animal methods are inherently time-consuming, expensive, and subject to the natural variability of biological response among animals.

Chemical and microbiological methods have provided improved reproducibility and generally much faster response at less cost. Microorganisms measure a microbiological response, but chemical methods measure chemical compounds, not "vitamin activity." Therefore, the accuracy of chemical methods, in terms of the measurement of biological effectiveness, depends on a knowledge of the vitamin composition and the ability to measure the appropriate chemical compounds.

Chemical detection methods may suffer from specificity problems—generally from too little but sometimes from too much. Chemical methods usually measure some molecular functional characteristic. This characteristic may be shared by compounds which do not have biological activity and which thus interfere or produce a positive bias. Such is the case with the antimony trichloride color reaction for vitamin A. Chemicals which have structural characteristics similar to vitamin A but do not have biological activity may respond to this color test for unsaturation. In these cases additional techniques may be employed to mask the interferent. The use of maleic anhydride to tie up unwanted *cis*-isomers is an example. Alternatively, additional steps may be taken to separate interferents (such as the use of column chromatography to remove carotenoids and other unknown interferences). Chemical methods for fat-soluble vitamins in natural product-based items such as infant formula nearly always include an initial extraction step to remove most of the multitude of nonvitamin constituents naturally present. Some of them, if not removed, are likely to interfere with any generalized chemical detection scheme.

On the other hand, several chemical compounds present in natural ingredients may have vitamin activity and not be measured. Separation steps used to eliminate interferences may remove compounds which do have some vitamin activity. For example, some of the carotenoids removed by column chromatography have vitamin A activity. In these cases, not all biological activity is measured and the method may be considered too specific. Since related isomers and derivatives are very likely to have differing biological activities, it can be a complex issue. Fortunately, the natural ingredients in infant formula are not expected to furnish the bulk of vitamin activity. Vitamins added to formula are of well defined composition and can be measured accurately, and only the smaller, natural levels present in raw materials are subject to this type of limitation.

In some cases it can be very useful for the analyst to know the composition of added vitamins. For example, vitamin A is readily available as either the all-*trans* or a 13-*cis*/*trans* mixture. Biological potency of the 13-*cis*-isomer is approximately 75% of the all-*trans*-isomer. Suppose a manufacturer buys and uses the cis-trans mixture of vitamin A. Analysts within the company are aware of the composition used, but an independent analyst might not be. If the total of *trans*- and *cis*-isomers is measured, the difference in biological activity of the 2 isomers may be ignored. Ideally the analyst would measure each individual isomer, but an officially recognized method for distinguishing vitamin A isomers in infant formula is currently not available. This simply illustrates that caution must be exercised in analyzing and interpreting test results for products whose composition is unfamiliar to the analyst/evaluator.

Chemical methods used today are quite reliable. Speed and reproducibility are orders of magnitude better than those obtainable by older animal methods. However, most current methods are still tedious and time-consuming. They can be much improved by the application of modern technology. Efforts are being made to develop a new generation of methods for various types of nutrients. However, changing to a different type of method may introduce new information about the nutrients in products.

Ideally, the development of a new method would immediately lead to analytical results identical to those from the current method. This frequently does not occur when a new method is first used; the results often differ somewhat. It may be that the proposed new method has some errors or biases which have not yet been uncovered and further work is needed. It may be, as we have found, that the new method has greater separating power so that new information becomes available. The underlying assumptions of what chemical compounds are to be measured must be reconsidered. Because of possible biases and interferences that are not detectable on the basis of a few samples and because of new information generated, new methods are carefully evaluated within the laboratory and by collaborative studies among companies and other interested parties such as the National Bureau of Standards and FDA. In the end, it may be concluded that results from the newer method are slightly different but better: The assumptions are better defined; the appropriate chemical compounds are being measured; and the results are more reflective of actual nutrient content. When the new method is employed, the historical data on the product may shift. In such cases the ongoing evaluation of products in stability studies must recognize and take into account the new information.

When new methods are developed that use powerful separating techniques such as high performance liquid chromatography, one frequently finds that a number of vitamin isomers appear. The presence and relative abundance

of these isomers may be new information. It is often desirable to quantitate these isomers. Unfortunately, reference materials may not be available, as is the case for several isomers of vitamins A, K, and E. Obviously, the lack of suitable reference materials provides a barrier to the improvement and implementation of better methods.

Methodology Trends

Let us focus our attention on some coming trends in analytical methodology. As we review these, keep in mind that improvements in method accuracy and reliability increase our ability to evaluate and predict nutrient content and thus assist us in designing and producing products with greater certainty of meeting product label claims.

Some ways in which we would like to improve test methods, along with some examples of technology which can provide such improvements, are as follows: accuracy and specificity (HPLC methods for vitamins); speed (infrared methods for protein, fats, and solids); automation (automated sampling and sample preparation; computer data acquisition and reduction); turnaround time and broad applicability (ICAP methods for inorganics); ruggedness and reliability (the descendents of the above).

More accurate and specific chemical methods for vitamins D and K are becoming available as a result of the work of Barnett and others who have published HPLC separations of these vitamins in infant formula products (1). The separating power of HPLC often yields separate, distinct peaks for various isomers of the fat-soluble vitamins, providing the opportunity of measuring isomers individually. In general HPLC methods are considerably faster than the methods they replace, even though sample workup still involves hydrolysis, extraction, and evaporation steps. Sometimes isomers are incompletely resolved. In such cases, measurement of overlapping peaks becomes awkward. Some separations are highly dependent on strict reproducibility of columns. In our experience, work has been delayed several months for the lack of columns identical to earlier columns on which development work was carried out. Again, the measurement of individual isomers is dependent on reference materials, some of which are not available.

Current analyses routinely used are considered accurate and reliable but the laboratory would benefit considerably if faster methods could be used, e.g., measurement of protein, fat, and solids. Instruments such as the InfraAlyzer®, sold by the Technicon Corporation, are capable of making such measurements very rapidly through measurement of infrared backscatter at several wavelengths. With appropriate calibration from a series of knowns, this instrument can compare a sample with its microprocessor-stored information and yield results in just a few seconds per sample. Several parameters can be determined simultaneously. This greatly increases the number of samples which can be tested daily in the laboratory. Results with this instrument are highly reproducible as long as the matrix remains constant. Since it is basically a comparative technique, frequent checks against established methods are in order. Separate calibrations, consisting of several samples covering a range of concentrations, are required for each separate formulation. The tendency is to become so dependent on the equipment that it is very difficult to continue using manual methods if the instrument is not functioning.

Laboratory efficiency can often be improved by appropriate automation. Our laboratory is in the initial phases of interfacing a Hewlett-Packard Laboratory Automation System to a variety of instruments. This minicomputer system will keep logs of the sample runs, obtain data from the instrument detectors, calculate and issue results, and keep files of the data. It will relieve much manual record keeping, peak measuring, calculating, and data handling. Eventually, we expect significant manpower savings. The interfacing of instruments to the computer requires a large investment in personnel training and programming time. Again, because it will handle so many routine functions, it may be difficult, but important, to maintain alternative manual capability in case of instrument failure.

Another branch of efficiency improvement through automation is the use of automated sampling systems. Autosamplers are becoming routine on chromatographs and other instruments throughout industry. People in our organization and elsewhere have been working on systems to perform sample workup in addition to simple sampling. Our work, which is in the preliminary stages at this point, is designed to use continuous flow systems to perform digestion and extraction steps followed by direct interfacing to a high performance liquid chromatograph for analysis of fat-soluble vitamins. Such an approach is designed to avoid the tedious manual workup steps and should result in

substantial economies. This approach obviously requires extensive development effort.

Any technology which can perform several tests simultaneously or decrease the turnaround time on samples is likely to generate considerable interest among laboratory managers. For some time our laboratory has been using a complex continuous-flow system designed and built by Barnett and his associates (2) for the simultaneous determination of 9 inorganic nutrient elements. This system is based on colorimetric and flame photometric chemistry and employs several pieces of Technicon® hardware. It has provided remarkable time savings and precision improvements over traditional methods. However, it requires considerable operator attention to maintain reagents, manifold conditions, and flow patterns.

In general, inorganic elements can also be measured by atomic absorption spectroscopy and inductively coupled argon plasma spectroscopy. These instruments are not new and are increasingly coming into routine use. Automatic sampling devices substantially reduce operator-attended time. Our effort at present is to develop techniques to avoid the preliminary ashing of samples. If this can be accomplished, substantial time savings and much faster sample turnaround can be realized.

These examples have been shown to be technically achievable and are only a few of the many trends we may expect to be developed in analytical laboratories over the next several years to make nutrient analysis faster and more reliable. As new methods are developed and validated through extensive collaborative studies, an evolutionary experience accompanies each one. This includes training of analysts and a fair amount of trouble-shooting. It takes time, frequently several months, for new technology to be thoroughly understood and mastered, and for the weak and sensitive places to be detected and strengthened. Over a period of years the second and third generations of new techniques become recognized as rugged and reliable standard laboratory methods. This evolution is painful, and it requires understanding, patience, and determination to bring it to fulfillment.

Reference

(1) Barnett, S. A., Frick, L. W., & Baine, H. M. (1980) *Anal. Chem.* 52, 610–614

(2) Barnett, S. A., Hart, L. J., Baine, H. M., & Joiner, C. B. (1981) 95th Annual Meeting of the AOAC, Oct. 19–22, Washington, DC

Nutritional Adequacy of Commercial Baby Foods

D. L. YEUNG
H.J. Heinz Company of Canada Ltd., 250 Bloor St E, Toronto, Ontario, Canada, M4W 1G1

The nutritional adequacy of commercial baby foods is assessed from data derived from a longitudinal nutrition survey of 400 infants in Canada. On the average, the nutrient intakes from infant food meet or exceed the recommended daily allowances for most nutrients. Most infants would not be able to meet the minimum daily requirements for iron, B vitamins, or calcium if infant cereals were not fortified.

Commercial baby foods are intended as transitional foods between a diet comprised solely of breast milk or formula or both, and a diet consisting of table foods or foods that are prepared for consumption by all members of the family. Baby foods are generally introduced when the infant is 3–4 months old or when it is judged that the infant is no longer satisfied with milk alone. Thus commercial baby foods must be of high nutritional quality to complement milk so as to form a balanced diet for the growing infant.

The purpose of this paper is to provide a perspective on the nutritional adequacy of commercial baby foods. Two aspects of the baby foods will be discussed: (1) their contribution to the nutrient content of the infant's diet, and (2) the nutritional significance of nutrient fortification.

The nutritional adequacy of commercial baby foods is assessed as part of the infant's total diet, which is compared with an established dietary standard. This is appropriate because the recommended daily allowances are set to meet the nutritional requirements of the majority of healthy individuals. Baby foods are formulated according to these standards.

In this paper, the data presented are derived from a longitudinal nutrition survey of 400 infants, recently completed in Canada (1). The Canadian (2) and U.S. Dietary Standards (3) are slightly different for a small number of nutrients (Table 1). Foods in the 2 countries are fortified accordingly. For example, the RDA for iron for infants in the United States is 10–15 mg, and in Canada it is 7–8 mg. Infant cereals are fortified at 50 and 30 mg per 100 g in the United States and Canada, respectively. Although the ratios between the RDA and levels of fortification in the 2 countries are not identical, they are close. In addition, the varieties of baby food and the nutrient contents of the nonfortified baby foods in Canada and the United States are similar. Hence the use of the Canadian data is suitable for the task at hand. Furthermore, the results are not derived from artificially constructed diets. They represent actual infant feeding practices.

Commercial baby foods are popular. Nearly all infants consume some commercial baby foods during infancy (Figure 1). During the peak period of consumption, which is about 5–8 months of age, over 95% of infants eat commercial baby foods.

On the average, the nutrient intakes of infants meet or exceed the recommended daily allowances, which is referred to in Canada as the RDI (Figure 2). During the first 18 months of life, the mean vitamin intakes exceed the RDI. The intakes of vitamin A and C are well above recommendation. One of the main reasons for the high intake of these 2 vitamins is vitamin supplements, which are given to over 75% of infants. They provide about 30% of the daily vitamins A and C. However, because the intakes of the 2 vitamins from foods exceed the RDI, vitamin supplements are largely unnecessary.

With the exception of calcium and iron at 1 month and the iron intake at 18 months, the

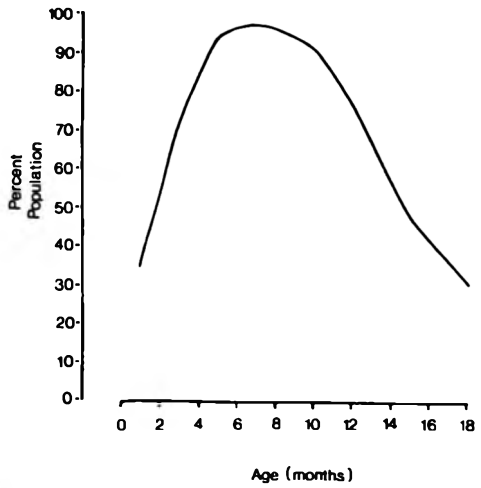


Figure 1. Percent of population using commercial baby foods.

Presented at the Symposium on Infant Formula Regulation and Infant Food Problems, 95th Annual Meeting of the AOAC, Oct. 19–23, 1981, at Washington, DC.

0004-5756/82/6506-1500–05$01.00

Table 1. Recommended daily nutrient intake in the United States and Canada

Age, year	Wt, kg	Ht, cm	Protein, g	Ca, mg	P, mg	Fe, mg	Vit. A, RE	Thiamin, mg	Riboflavin, mg	Niacin, NE	Vit. C, mg
0–0.5:											
U.S.	6	60	kg × 2.2	360	240	10	420	0.3	0.4	6	35
Canada	6	—	kg × 2.2	500	250	7	400	0.3	0.4	5	20
0.5–1.0:											
U.S.	9	71	kg × 2.0	540	360	15	400	0.5	0.6	8	35
Canada	9	—	kg × 1.4	500	400	7	400	0.5	0.6	6	20
1.0–3.0:											
U.S.	13	90	23	800	800	15	400	0.7	0.8	9	45
Canada	13	90	22	500	500	8	400	0.7	0.8	9	20

mean mineral intakes exceed the RDI (Figure 3). From about 10 months onwards, dietary sodium is 300% or more of RDI. The primary source of sodium is table foods that contain either table salt or salted canned foods.

When the dietary intakes of infants who were exclusively fed commercial baby foods and milk are compared with the RDI, the diets are shown to be nutritionally adequate (Table 2). For this purpose, only data at 5 and 8 months are shown. It is apparent that the nutrient contents of the diets are above recommended levels.

In any dietary study, the final analysis of the adequacy of the diet is the growth of the subjects (Figure 4). Comparison of the mean body weights of the male and female infants with the growth standard of the National Center for Human Statistics (4) shows that they are close to the 50th percentile. Hence the infants are eating enough to achieve normal growth. The mean weights of the 5- and 8-month-old infants who were exclusively eating commercial baby foods and milk were 7.19 and 8.59 kg, respectively. These are again within normal ranges of weight published by NCHS. This indicates that indeed commercial baby foods do complement milk to provide a nutritionally adequate diet to maintain normal growth.

Commercial baby foods are important sources of the infant's daily nutrient intakes (Table 3). During the peak period of consumption, i.e., from 5 to 8 months, commercial baby foods contribute slightly over 20% of vitamin C, 32% of vitamin A and riboflavin, and over 50% of thiamin and niacin in the diets of the study population. Commercial baby foods contribute minimally to the sodium intake, i.e., only about 17%. They provide less than 20% of dietary potassium and about a quarter of the calcium and phosphorus in the diet. However, they are the primary source of iron, providing about 80% of the dietary iron.

From the foregoing results, it may be concluded that in the general population where most infants are eating commercial baby foods as well as home-prepared foods and milk, the former are excellent or good sources of most nutrients except sodium, potassium, and fat.

Commercial baby foods consist of infant cereals, fruit juices and drinks, strained and junior vegetables, fruits, meats, meat dinners, vegetable and meat combinations, and desserts. Among

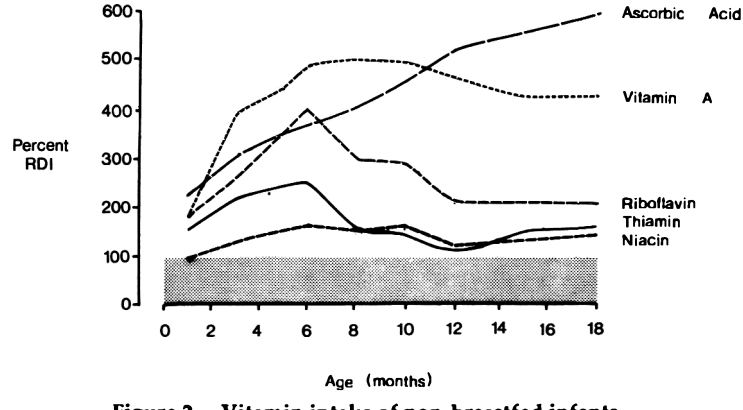


Figure 2. Vitamin intake of non-breastfed infants.

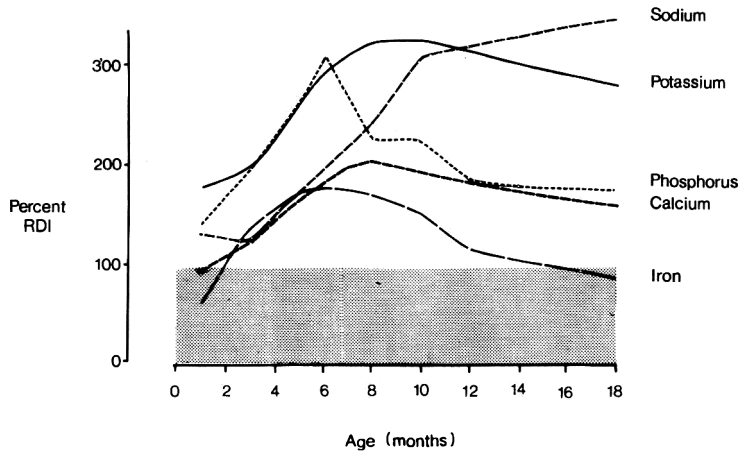


Figure 3. Mineral intake of non-breastfed infants.

the numerous varieties, only the infant cereals and fruit juices are fortified. Infant cereals are fortified with iron, thiamin, riboflavin, niacin, and calcium. Baby fruit juices and drinks (except orange) are fortified with vitamin C.

To evaluate the adequacy of fortification of commercial baby foods, infant cereals will be used as an example. The incidence of adequacy of intake of the various nutrients among the infants is examined under 2 conditions, i.e., (1) the infant cereals are fortified and (2) they are not fortified.

Infant cereals are fortified with 30–50 mg of the highly bioavailable electrolytic iron per 100 g. Electrolytic iron supplies over 80% of the total dietary iron from 5 to 8 months of life. There is a strong statistical correlation between total intake of iron and the intake of infant cereals (Figure 5). As the average intake of infant cereals decreases, there is a corresponding decrease in dietary iron and an increase in the number of infants not meeting their recommended iron intake. In fact, without fortification of infant cereals, the RDI for iron is difficult to meet.

To evaluate the nutritional significance of fortifying infant cereals with calcium, thiamin, riboflavin, and niacin, the intakes of the nutrients by infants are assessed according to the standards used in the Nutrition Canada Survey (5). To simplify data presentation, only the data for 6-month-olds are shown. The choice of 6 months is appropriate because by this time almost all infants are eating infant cereals.

At 6 months, the mean dietary calcium intake is 792 mg. If infant cereals were not fortified, the mean intake would be 623 mg. The RDI of 500 mg is exceeded in both instances. But when the incidence of adequacy of calcium intake is assessed according to Nutrition Canada, a different picture emerges (Table 4). The number of infants receiving inadequate and less-than-ade-

Table 2. Nutrient intake of infants exclusively fed commercial baby foods and milk

Nutrient	RDI, %	
	5 months of age	8 months of age
Protein	143	299
Calcium	157	222
Phosphorus	256	277
Iron	176	176
Sodium	161	239
Potassium	238	321
Vitamin A	414	467
Thiamin	247	168
Riboflavin	345	333
Niacin	155	147
Ascorbic acid	358	400

Table 3. Percent nutrients contributed by commercial baby foods in diets of infants from 5 to 8 months old

Nutrient	Percent from commercial baby foods
Protein	21.4
Fat	11.1
Carbohydrate	36.5
Vitamin A	32.4
Thiamin	52.3
Riboflavin	32.2
Niacin	55.6
Ascorbic acid	22.5
Calcium	22.6
Phosphorus	24.5
Iron	80.8
Sodium	17.4
Potassium	19.2

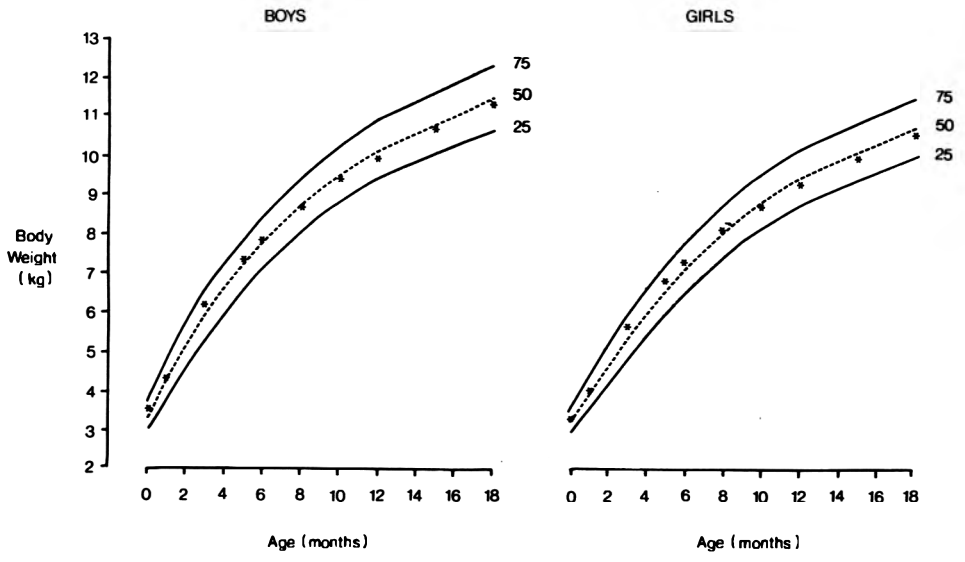


Figure 4. Weight of infants in relation to National Center for Health Statistics weight curve.

quate amounts of calcium is initially small. However, if the infants ate identical amounts and types of foods, with the exception that infant cereals were not fortified, the incidence of inadequate calcium intake increases 4.5 times, and the incidence of less-than-adequate intake doubles. In total, about one quarter of the infant population receive inadequate calcium intake if infant cereals are not fortified with calcium.

The 1:1 ratio of Ca:P is essential for optimum utilization of calcium by the infants. The Ca:P ratio in the diets of infants who consume adequate amounts of infant cereals is at or above 1:1. However, infants who do not consume fortified infant cereals but instead consume adult-type cereals are at risk of having diets with a Ca:P ratio of less than 1:1. This further supports the nutritional value of calcium fortification of infant cereals.

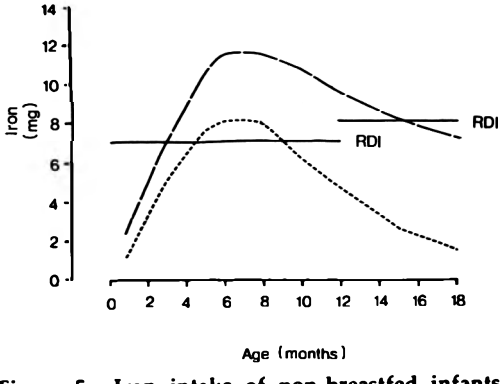


Figure 5. Iron intake of non-breastfed infants: dashed line, dietary iron; dotted line, infant cereal iron.

Fortification of infant cereals with thiamin, riboflavin, and niacin was similarly evaluated. The incidence of adequacy of intake of the vitamins determined according to Nutrition Canada is given in Table 5. As the table shows, removal of the B vitamins from the infant cereals, especially thiamin and niacin, increases the risk of inadequate intake of the vitamins.

From the foregoing, it may be concluded that the fortification of infant cereals with iron, calcium, and the B vitamins is indeed beneficial to a large number of infants.

The variety of baby foods is modest. There is no attempt to ensure that each variety is in itself nutritionally complete because baby foods form only a part of the infant's total diet. The choices allow expression of individual preferences in

Table 4. Effect of cereal fortification on dietary calcium status of non-breastfed infants at 6 months of age

Calcium status	Percent population	
	Infant cereals fortified[a]	Infant cereals not fortified
Inadequate (<400 mg)[b]	2.9	14.3
Less than adequate (400–500 mg)	5.7	11.5
Adequate (>500 mg)	91.4	74.2

[a] Infant cereals fortified with 1075 mg calcium/100 g.
[b] Nutrition Canada.

Table 5. Effect of cereal fortification on thiamin, riboflavin, and niacin of non-breastfed infants at 6 months

	Inadequate		Less-than-adequate		Adequate	
Nutrient	+ Cereal	− Cereal	+ Cereal	− Cereal	+ Cereal	− Cereal
			% Population			
Thiamin	0.4	19.6	6.1	38.9	93.6	41.4
Riboflavin	0.4	1.1	0.7	8.2	98.9	90.7
Niacin	15.4	50.0	22.9	22.5	60.7	27.5

tastes and flavors. A diet consisting of a variety of foods encourages new experiences and avoids monotony in the diet that could result in excessive or deficient nutrient intake.

Evaluation of baby foods by data obtained from nutrition research confirms the importance of commercial baby foods in infant nutrition. The results also show that they do indeed complement milk in providing infant diets that are adequate to maintain normal growth.

The importance of fortification of infant cereals is clear. Without iron-fortified infant cereals, most infants would have difficulty satisfying the RDI. Fortification of infant cereals with calcium, thiamin, riboflavin, and niacin substantially reduces the incidence of inadequate intake of the nutrients.

One of the criteria for formulating baby foods is nutrition. This is guided by research and by published literature information. The nutrient content of baby foods and the form and level of nutrient fortification are continuously assessed. A case in point is iron fortification of cereals. Before 1977, the form of iron fortification was sodium iron pyrophosphate at 100 mg per 100 g of cereals. Since this form of iron was found to be of low bioavailability, the form of iron was changed to electrolytic iron of small particle size, which is 2–4 times as available as the pyrophosphate. Consequently, the level of fortification was reduced to 50 mg in the United States and 30 mg in Canada.

The nutritional quality of baby foods is sound according to today's knowledge of infant nutrition. As new knowledge becomes available and if it is deemed scientifically advisable, the baby foods industry will act accordingly to ensure optimal nutrition for infants.

References

(1) Yeung, D. L., Leung, M., Hall, J., & Medina, D. (1979) *J. Can. Diet. Assoc.* **40**, 228

(2) Dietary Standard for Canada (1975) Bureau of Nutritional Sciences, Food Directorate, Health Protection Branch, Department of National Health and Welfare, Canada

(3) Recommended Daily Dietary Allowances (1980) Food and Nutrition Board, National Academy of Sciences-National Research Council, Washington, DC

(4) Hamill, P. V. V., et al. (1979) *Am. J. Clin. Nutr.* **32**, 607

(5) Nutrition Canada, National Survey (1973) Department of National Health and Welfare, Canada

Infant Food Problems and Analytical Aspects of Providing Safe and Wholesome Infant Foods

R. B. READ, JR
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

The technology of milk and infant food production, storage, and handling has advanced to the point that infant foods have had an excellent record of being free of recognized health hazards. The Infant Formula Council has published methods specifically for these foods. Methods are included from *Standard Methods for the Examination of Dairy Products, Official Methods of Analysis, Bacteriological Analytical Manual*, the *Manual of Clinical Microbiology*, and the *U.S. Pharmacopeia*. Criteria for microbiological quality have been issued by the Codex Alimentarius Commission, the International Commission for Microbiological Specifications for Foods, and the Public Health Service. Surveys in the United States and Canada have shown that sanitary standards are met with ease.

With the increased employment of women in the late 19th century, the use of milk-based infant foods as a substitute for breast feeding became common. Unfortunately, the technology of milk production, storage, and handling had not advanced so that this shift could be accomplished without significant impact on infant health. At the turn of the century, infant mortality was described as a class mortality in the United States—highest in cities and towns where women worked in industrial establishments and put their children "early to bottle." In the United States in 1905, about one-fifth of deaths of people of all ages from all causes were of infants under 1 year of age. Of the infant deaths, over 40% were from diarrhea and enteritis—diseases thought to be foodborne in origin.

Milk offered for sale in Washington, DC, was described "for the most part (as) old, warm, and dirty." Examination of milk made at the Hygienic Laboratory of the Public Health and Marine Hospital Service during the summer of 1906 showed an average aerobic plate count of 22 million bacteria per mL compared with our maximum limit of 20,000 bacteria per mL today. Another study in 1906 of 272 samples from 104 dairies supplying milk to Washington, DC, found that 7% of these samples contained tubercle bacilli virulent for guinea pigs. Milkborne epidemics of typhoid, scarlet fever, and diphtheria were common. Vendors of milk in some cases could not get the milk to the consumer's doorstep without it spoiling, even with twice-a-day home delivery (1).

Obviously, the combination of a severe health problem coupled with an economic problem caused great concern that ultimately led to the use of sanitary practices, refrigeration, and pasteurization, with the result that one of our formerly most dangerous foods has become one of the safest.

Reported Microbiological Problems With Infant Foods

In the last 10 years, there have been few reports of microbiological problems with infant foods. The Centers for Disease Control have no record of infant foods causing illness. We have found 6 lots of contaminated infant formula of which 2 were reported to cause illness. Our findings were *Pseudomonas aeruginosa* in a canned soy-base liquid infant food concentrate in 1972; staphylococcal enterotoxin A in a milk-base ready-to-eat infant formula in 1976; 3 lots of milk-base powder for reconstitution contaminated with *Klebsiella pneumoniae* or *Escherichia coli* in 1979; and one lot of liquid milk-base concentrate containing thermophiles in 1981. The illnesses reported were from the lots that contained staphylococcal enterotoxin A and *K. pneumoniae*.

In 1976, infant botulism was first recognized. Its appearance raised questions as to the source of *C. botulinum* spores which infected some infants. *C. botulinum* is primarily found in the soil. Of 250 soil samples taken from throughout the United States, 62, or 24%, were found to contain this organism (2). Because of soil contamination, one would expect to find a low level of *C. botulinum* spores or vegetative cells in most foods that did not receive a process to eliminate this organism such as that used for low acid canned foods.

Of the unsterile infant foods that have been analyzed for *C. botulinum*, honey has received the most attention. A survey of honey was made in California which showed that 10% of the samples

Presented at the Symposium on Infant Formula Regulation and Infant Food Problems, 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

0004-5756/82/6506-1505–04$01.00

contained *C. botulinum* (3). Sugiyama and coworkers in Wisconsin (4) found that 3.7% of 55 retail samples of honey contained *C. botulinum*, whereas 9% of the producers' samples they tested contained this organism. We tested 100 samples of honey as a part of a general survey of infant foods and found 2 to contain *C. botulinum*.

In addition to the honey tested in our survey, we tested approximately 100 samples each of cooked fresh carrots, dry infant cereal, commercially canned fruits, corn syrup, dry commercial baby formula, regular pasteurized cow's milk, nonfat dry milk, and sugar. None of these foods yielded *C. botulinum* except corn syrup; *C. botulinum* was found in 8 of the first 40 samples of corn syrup tested. This prompted a national survey of corn syrup involving 960 samples. Five bottles were found to contain *C. botulinum*.

The finding of *C. botulinum* spores or vegetative cells in foods other than low acid canned foods is expected and cannot be considered to be the result of a defective process or improper care of the food after processing. The significance of the occasional low level of this organism in infant foods remains to be determined because infants susceptible to infant botulism will receive the organism from their environment as well as from their food supply.

Analytical Methods

The Infant Formula Council has published methods specifically for these foods (5). These include methods for the standard plate count, coliform count, thermophile count, yeast and mold count, coagulase-positive staphylococci, *Escherichia coli*, *beta*-hemolytic streptococci, and *Salmonella* as well as methods for sterility testing. The first 4 methods are similar to those in *Standard Methods for the Examination of Dairy Products* (6). The subsequent methods are selected from such sources as the *Official Methods of Analysis* of the AOAC (7), the *Bacteriological Analytical Manual* of the FDA (8), the *Manual of Clinical Microbiology* of the American Society for Microbiology (9), and the *U.S. Pharmacopeia* (10). The Infant Formula Council manual was published in 1972 and has not been updated. As a result, there are minor differences between the methods recommended by the Council and those recommended in current editions of those publications cited by the Council as their source of methods.

Bacteriological methods for the examination of prepared infant formula have been published by the Public Health Service (11). The methods advocated are also similar to those found in *Standard Methods for the Examination of Dairy Products*.

Microbiology of Infant Formula

Two major studies of the microbiological quality of dry milk mix and milk substitute infant formula have been done recently, one in Canada by the Food and Drug Directorate (12) and the other by the U.S. Food and Drug Administration. Both studies were designed so that the results would be representative of the nation. Generally, the microbiological quality of infant formula was excellent, e.g., in the U.S. study, the geometric mean aerobic plate count of the milk-containing products was 25/g, whereas the mean for milk substitute products was 52/g. Milk-containing products sampled in the United States had a lower aerobic plate count than those sampled in Canada. In the United States, 77 and 96% of the products had aerobic plate counts of $\leq$50/g and $\leq$500/g, respectively, whereas the Canadian percentages were 9 and 62. This rather large difference was reversed in part in the APC values for milk substitute products. With these in the United States, 60 and 90% were $\leq$50/g and $\leq$500/g, respectively, whereas in Canada, the percentages were 93 and 100.

Very few data have been reported for infant foods from countries other than the United States and Canada. One report has been published of an Indian study in which samples of infant food were examined, one each of 7 different brands of infant milk-based product and 3 brands of milk-cereal products (13). Although the APC values of 2×10^3 to 9×10^3/g were higher than most sampled in the United States and Canada, the values are either within or do not greatly exceed those recommended for infant formula in international trade.

Microbiological Criteria for Infant Foods

Infant formula and other infant foods are perceived by most as requiring special consideration because of the generally susceptible population at risk. This is reflected in the interest that various groups have shown in setting microbiological criteria for these foods as well as in the rather strict criteria that have been recommended.

The Codex Alimentarius Commission of the Food and Agriculture Organization of the United Nations, World Health Organization, has prepared a "Recommended International Code of Hygienic Practice for Food for Infants and Children" (14). This code of practice is advisory in

Table 1. Codex microbiological specifications for foods for infants and children

Product	Test	n^a	c^b	Limits per g m^c	M^d
Dried biscuit type					
Plain	none	—	—	—	—
Coated	coliform	5	2	<3	20
	Salmonella	10[e]	0	0	—
Dried and instant	APC	5	2	10^3	10^4
(not heated before	coliform	5	1	<3	20
eating)	*Salmonella*	20	0	0	—
Dried; heated before	APC	5	3	10^4	10^5
eating	coliform	5	2	10	100
	Salmonella	5	0	0	—
Thermally processed in hermetically sealed containers		shall be free of toxins and commercially sterile.			

[a] Number of samples to be tested.
[b] Maximum allowable units of marginal quality.
[c] Value which separates good from marginal quality.
[d] Value which separates marginal from defective quality.
[e] Each sample unit tested is 25 g.

nature and it is left to individual governments to decide what use they wish to make of the code.

Microbiological specifications have been included in this Code of Practice for foods for infants and children up to 3 years. The foods covered are dried biscuit-type products, dried and instant products intended for consumption after the addition of liquid (including dried infant formulas and instant infant cereals), dried products requiring heating before consumption, e.g., sweetening agents, starches, texturizers and similar products, and, finally, thermally processed products packaged in hermetically sealed containers. The latter category includes canned products and liquid infant formulas. The specifications include those for APC, coliform, and *Salmonella*. These are given in Table 1.

The International Commission for Microbiological Specifications for Foods also has recommended microbiological criteria for special dietary foods, which include foods for infants (15). Specifications are stipulated for APC, *E. coli*, *S. aureus*, *B. cereus*, *C. perfringens*, and *Salmonella*. The criteria recommended are shown in Table 2.

Table 2. Microbiological limits recommended by the International Commission on microbiological specifications for foods for special dietary foods including foods for infants

Test	n^a	c^b	Limits per g m^c	M^d
APC	5	1	10^4	10^6
E. coli	5	2	<3	10
S. aureus	10	1	10	10^2
B. cereus	10	1	10^2	10^4
C. perfringens	10	1	10^2	10^3
Salmonella	60[e]	0	0	—

[a–e] See Table 1.

The Public Health Service has published "Minimum Sanitary Standards for Community Infant Formula Services" (11), which are directed towards the preparation, heat treatment storage, and distribution of prepared infant formula. This standard requires taking a minimum of 3 samples from each autoclave load so as to be representative of the heat treatment in different locations within the autoclave. Microbiological criteria on the finished products are based on the arithmetic average of the samples from each lot. The criteria are as follows: The average APC for heat-treated formula shall not exceed 25/mL and the coliform count shall not exceed 1/mL for the lot to be satisfactory. If either or both of these values are exceeded, the standard requires that the lot be destroyed.

In summary, infant foods have had an excellent record of being virtually free of recognized health hazards, which is a tribute to the industry that produces these foods. This record can only be maintained with the continued use of high standards governing the production, processing, and distribution of these foods under hygienic conditions. The rare finding of contaminated infant foods serves as a warning as to what can happen when standards are relaxed. Our analytical methods for the determination of microbiological content of infant foods appear adequate and quite well standardized on a national

and international basis. Those who have recommended that microbiological criteria be set for infant foods have set high standards for these foods. Our assessment of the performance of the infant food industry indicates that these standards are being met with ease by the industry in the United States.

REFERENCES

(1) Rosenau, M. J. (1909) Milk and its relationship to public health. *Hygienic Laboratory Bulletin No. 56*, U.S. Government Printing Office, Washington, DC

(2) Smith, L. (1978) *Health Lab. Sci.* **15**, 74–80

(3) Midura, T. F., Snowden, S., Wood, R., & Arnan, S. (1979) *J. Clin. Microbiol.* **9**, 282–283

(4) Sugiyama, H., Mills, D., & Kuo, L. (1978) *J. Food Prot.* **41**, 848–850

(5) Infant Formula Council (1972) *Microbiological Methods for Analysis for Infant Formulas*, Chicago, IL

(6) Marth, E. H. (Ed.) (1978) *Standard Methods for the Examination of Dairy Products*, 14th Ed., American Public Health Association, Washington, DC

(7) *Official Methods of Analysis* (1970) 11th Ed., AOAC, Arlington, VA

(8) Food and Drug Administration (1969) *Bacteriological Analytical Manual*, 2nd Ed., Washington, DC

(9) American Society for Microbiology (1970) *Manual of Clinical Microbiology*, Ann Arbor, MI

(10) *United States Pharmacopeia* (1970) 18th Rev., U.S. Pharmacopeial Convention, Rockville, MD

(11) U.S. Department of Health, Education, and Welfare (1967) Minimum Sanitary Standards for Community Infant Formula Services. *Public Health Service Publication No. 1625*, U.S. Government Printing Office, Washington, DC

(12) Collins-Thompson, D. L., Weiss, K. F., Riedel, G. W., & Charbonneau, S. (1980) *J. Food Prot.* **43**, 613–617

(13) Singh, R. S., Singh, S., Batish, V., & Ranganathan, B. (1980) *J. Food Prot.* **43**, 340–342

(14) Codex Alimentarius Commission (1979) *Recommended International Code of Hygenic Practice for Food for Infants and Children*, Food and Agriculture Organization of the United Nations, Geneva, Switzerland

(15) International Commission for Microbiological Specifications for Foods (1974) *Microorganisms in Foods: Sampling for Microbiological Analyses: Principles and Specific Applications*, University of Toronto Press, Toronto, Ontario, Canada

The Infant Formula Industry, the Infant Formula Act of 1980, and Quality Control

ROBERT C. GELARDI
Infant Formula Council, 5775 Peachtree-Dunwoody Rd, Atlanta, GA 30342

Throughout the months following the discovery of the low-chloride problem in infant formula, the industry undertook independent and voluntary measures to ensure the compositional quality of formulas. Members of the Infant Formula Council reviewed industry procedures, practices, and policies, and submitted a detailed description of its quality control and clinical testing procedures to the Food and Drug Administration. New product concepts and product reformulation proposals are based on medical and nutritional findings, on changes in medical concepts for nutritional support, on availability of improved nutrient sources, or on improved ingredients or processing technologies. Quality control is maintained throughout the manufacturing process through inspection of raw materials, analysis of major and minor constituents, equipment and process controls, and continuous product monitoring. However, despite cooperation of the infant formula industry with FDA and Congress during the development of the infant formula bills, the proposed document was too rigid and did not take into account that manufacturing methods of a given manufacturer often vary for the same product, for different products, and on different production equipment. The statistical criteria established uneven and inconsistent requirements for various manufacturers. Congress did not forsee that the proposed regulatory procedures would significantly affect industry.

Organized in 1970, the Infant Formula Council today represents the United States manufacturers of infant formula who produce essentially all the infant formula in this country. The infant formula industry recognizes the unique place its products have in the critical initial development of millions of youngsters. For that reason, the industry is committed to providing the highest quality products possible. During the first decade following the Council's formation, almost all our work was scientific and technical and was carried out by the Council's 2 principal committees: the Committee on Nutritional Sciences and the Technical/Quality Control Committee.

Furthermore, the Council recognizes and acknowledges the importance of human milk and breast-feeding. The industry fully supports breast-feeding as the preferred feeding practice for babies and constantly works on improving its formulas to incorporate as much as possible the nutritional benefits provided by human milk. Formulas on the market today are designed to meet or exceed the nutritional standards of the Committee on Nutrition of the American Academy of Pediatrics.

During the industry's several decades of experience in manufacturing infant formula, it has consistently directed itself to assuring that only the finest quality products are provided to consumers. The rarity of problems which have developed during the past 60-plus years attests to its success.

But in 1979, unfortunately, an error did occur, resulting in a major recall of one manufacturer's product. In this paper, I will review industry involvement in events leading up to infant formula legislation, as well as the impact of the Infant Formula Act on quality control to date, and the potential future impact on the industry and consumers if the quality control regulations initially published by the Food and Drug Administration had been enacted as initially proposed.

Throughout the months following discovery of the low-chloride problem in 1979, the infant formula industry supported the efforts of the Food and Drug Administration to thoroughly examine industry practices and initiated voluntary measures to the same end.

In addition to FDA-initiated efforts to assure the compositional quality of infant formulas, the industry undertook independent and voluntary measures to achieve the same goal. Members of the Infant Formula Council conducted an extensive review of industry procedures, practices,

Presented at the Symposium on Infant Formula Regulation and Infant Food Problems, 95th Annual Meeting of the AOAC, Oct. 19–23, 1981, at Washington, DC. As originally presented, this speech focused, in large part, on the quality control regulations proposed by FDA on December 30, 1980. Subsequent to the speech and the Infant Formula Council's formal filing of similar comments with FDA, FDA (on April 20, 1982) published a revised regulation as a Final Rule which became effective on July 19, 1982. The FDA Final Rule incorporated most of the recommendations made by the Infant Formula Council. It is gratifying to note that responsible presentation of facts can impact on regulatory proceedings in a way which benefits both industry and consumers.

Note: Subsequent to this speech, all IFC members have agreed to include a single universal dilution symbol on all liquid concentrated formula and to include pictograms (showing proper preparation) on all forms of formula.

0004-5756/82/6506-1509-05$01.00

and policies to assure that the highest quality infant formulas possible were provided. As a part of this review, the industry submitted a detailed description of its quality control and clinical testing procedures to the Food and Drug Administration. In addition, an industry commitment was affirmed not only with respect to current and proposed FDA Good Manufacturing Practices and Low-Acid Canned Food Regulations, but also with respect to practices beyond those proposed.

Despite renewed efforts on the part of industry and FDA to assure that proper measures were in place to produce a safe and nutritious product, Congress believed that legislation was necessary to achieve that goal. Subsequently, hearings were held in both houses, at which I testified on behalf of the infant formula industry.

Traditionally, industry in general tends to take an anti-legislation posture. The infant formula industry, to the contrary, cooperated with Congress every step of the way as various infant formula bills were developed. This was recognized by former Senator Richard Schweiker (R-PA), who at the June 12, 1980 Hearing of the Subcommittee on Health and Scientific Research of the Committee on Labor and Human Resources stated, "I am impressed to see the industry come forward and endorse the regulatory bill. Not many industries have done that around here, even when it is serious, although there are obviously some problems with what is (sic) happening in the past. I do commend you for that position."

In this atmosphere of cooperation, Congress, industry, and the public were in close agreement with the objective of the Infant Formula Act: to assure that infant formula contains essential nutrients at appropriate levels. Thus, on September 26, 1980 the Infant Formula Act of 1980 was signed into law with the endorsement of the Infant Formula Council.

Intent of the Infant Formula Act

Under the provisions of this Act, the Food and Drug Administration was given the responsibility for establishing quality control procedures to assure that infant formulas comply with the Act. Specifically, Congress prescribed the boundaries of this authority, that is, the procedures may establish controls as necessary to assure that infant formulas contain appropriate levels of nutrients.

As stated in the Regulatory Impact Statement of the Senate Report, Congress was seeking to establish provisions for the manufacture and marketing of infant formula products which conformed to good business and scientific standards, and "which the regulated industry represents as being comparable to standards currently observed."

Obviously, Congress did not envision that these quality control procedures would have a dramatic impact on the infant formula industry which already subscribes to high standards of quality control.

Current Quality Control Practices

Industry's commitment to Good Manufacturing Practices (GMP) is evidenced by the rarity of problems which have developed during the 60-plus year history of infant formula production. Quality and appropriate nutrient composition are essential components of product development. Accordingly, infant formula producers emphasize product design and process control as well as specific composition monitoring.

Recognizing an obligation to keep high quality products economically affordable, infant formula producers have worked innovatively to assure that each batch of product has the proper content of each claimed nutrient substance. Systems which allow this goal to be accomplished are designed so that the addition of the proper quantity of each nutrient substance to each lot of product must be verified before the lot is released for distribution.

However, quality can be achieved only if it is carefully designed and built into the product. Therefore, each company bases new product concepts and product reformulation proposals on medical and nutritional findings, on changes in medical concepts for nutritional support, on availability of improved nutrient sources, or on improved ingredient or processing technologies. Product reformulation is initiated only after detailed nutrient specifications have been determined. The new or reformulated product is developed through laboratory and pilot plant experimentation. To assure that prescribed nutrient levels are being achieved, formulas are subjected to nutrient assays throughout the development phase. In addition, appropriate ingredient, stability, pre-clinical, and clinical tests are performed as necessary. The product must show satisfactory analytical tests and possess acceptable stability before its acceptance.

When a product is ready for manufacture, multiple steps in the infant formula manufacturing process are then subjected to rigorous

quality control procedures. Incoming raw materials are evaluated to ensure that they meet carefully defined standards of quality before use. Each company maintains and constantly updates its own list of approved vendors. Also, each carefully reviews any new vendor for any raw materials before the vendor is added to the company's list of approved suppliers of the item. Sources of protein, fat, and carbohydrate are added directly in the processing of each batch. Direct analysis of protein and fat in each lot provides verification that the proper quantities of these major constituents are present. The presence of the proper quantity of carbohydrate is generally calculated by mathematical differences based on a determination of total solids.

Constituents such as vitamins and minerals may be added as premixes. These premixes are prepared in bulk, and tested for the proper concentration and uniformity of mixing of each constituent substance. Presence of the proper quantity of these constituents is verified by analyzing each batch of product for one or more of these constituents, or by review of appropriate data to ensure that proper additions have been made.

In addition to the product formulation evaluation, the industry also uses equipment and process controls. Sterilization processes are developed from knowledge of the bacterial load in the unsterilized mixture and heat penetration studies of the product. These processes are specified in the Standard Manufacturing Plans and are executed by, or under the direction of, plant personnel thoroughly trained in Low-Acid Food Regulations. Before release, batch record auditors verify compliance with established procedure. In addition, samples of each batch are incubated and subsequently inspected.

Specifications and controls for nonproduct materials which directly affect product quality are established, used, and maintained by each company. These procedures assure that compressed air and air compressors, cooler water, nitrogen, approved boiler water additives, steam, and ingredient water are suitable for intended purposes. Similarly, suitable methods and materials are used for cleaning food contact surfaces.

Periodically through the shelf life of each product formulation, selected batches are subjected to detailed analysis. This policy provides continuing assurance that there are not undetected changes in processing, equipment, or material quality that might adversely affect the quality of the product after manufacture. These analyses ensure that the system is working as intended.

In summary, IFC members maintain quality control throughout the infant formula manufacturing process through inspection of raw materials, analysis of major and minor formula constituents, equipment and process controls and continuous product monitoring. Members of the Infant Formula Council each maintain a current written procedure which is used for infant formula products manufactured under their control, including a plan for initiating and effecting recalls or product withdrawals of any products, should they be indicated.

As specified under the provisions of the Infant Formula Act, until new FDA quality control regulations were effected, each infant formula manufacturer had to certify that infant formulas complied with the nutrient requirements of the Act and had to notify the FDA of this compliance every 90 days. Manufacturers failing to comply with nutrient requirements are subject to both civil and criminal penalties.

Industry Objections to the Proposed Quality Control Procedures

The unfortunate incident which led in part to the Infant Formula Act and current FDA quality control regulations arose from the well motivated decision on the part of one manufacturer to reduce sodium in its formula. Likewise, FDA's initially proposed quality control regulations contained many requirements which were new and untested and could similarly have led to adverse and unintended consequences.

FDA has always recognized that manufacturers do not employ identical manufacturing methods. This is true even with respect to manufacturers of seemingly similar products, such as infant formulas. Born of necessity, therefore, is the traditional philosophy underlying FDA quality control regulations that FDA will not tell a manufacturer how to reach an objective, but only that the objective must be reached.

Unfortunately, this sound and pragmatic philosophy was slighted in the proposed quality control regulations. The proposed document was very much a "how to" document that indicated a misconception regarding the variety of manufacturing methods employed within the infant formula industry. In fact, the manufacturing methods of a given manufacturer often vary for the same product, for different products, and on different production equipment. Forcing manufacturers into a single, set, untried

system is not only costly, but dangerous to quality maintenance and a barrier to improved quality and product innovation.

The burden the initial proposals would have imposed on the infant formula industry might have been justifiable if the public had received added assurance of the wholesomeness of infant formulas, but this was not the case. Rather, the proposed regulations, which required testing that has no relevance to given manufacturing processes, would have resulted in formula that is more expensive but not of higher quality.

As noted earlier, Congress did not foresee that the proposed regulatory procedure would significantly affect industry. Yet, the total cost to members of the Infant Formula Council of the FDA proposal could have easily exceeded $400,000,000 in the first 5 years. This includes $24,680,000 in capital expenditures and $70,940,000 a year in annual costs. These costs are very conservative. They would need to be increased considerably in actual practice, since all calculations exclude inflation, opportunity costs, profit, carrying costs for increased in-process inventory, and potentially extensive costs for unwarranted recalls and increased manufacturing capacity which eventually would have been required to offset the product arbitrarily rejected by inappropriate statistical criteria.

The statistical quality control criteria advanced in the proposed regulations were intended to ensure that infant formula provides the appropriate levels of nutrients as specified in the Infant Formula Act. However, the statistical criteria did not meet this goal, and furthermore, they would have established uneven and inconsistent requirements for the various manufacturers. Under the proposed system, nutrient acceptance ranges depended on process and raw materials variations, and on the relationship of the average to levels specified in the Infant Formula Act. The proposed regulations required corrective measures to be taken when a nutrient fell between the second and third standard deviation.

For example, if one manufacturer controls his vitamin A levels between 400 and 600 International Units, he would have had to destroy product below 400 and above 600 IU. If a second manufacturer controls his vitamin A levels between 300 and 700 IU, he would not have been required to destroy product until vitamin A was less than 300 or more than 700 IU. In both cases, the product might have been fit for use, but the manufacturer with a looser process would have been legally permitted to market the formula, whereas the manufacturer with the tighter process would have had to reject the formula. These examples illustrate that good and bad product cannot be defined based on where a point falls on a statistical control chart. This system would have been arbitrary and imposed a burden which would have served no apparent purpose. The statistical, arbitrary rejection rate would have ranged from about 4% upwards of 14% or more of all batches—even though all batches would have met all of the nutrient requirements of the Infant Formula Act. The application of the statistical criteria alone would have cost over $50,000,000 per year without any improvement in quality control.

Testing of the magnitude envisioned in the proposal may not have been possible and, if attempted, would have resulted in substantial, and potentially dangerous, processing delays. At a minimum, the microbial load would have been increased. Also, processing delays might have resulted in the loss of some labile vitamins. In-process analyses, therefore, would have quantitated some vitamins, the levels of which could have decreased by the time test results were known. The statistical criteria as well as the duplicative testing procedures did not provide assurance that infant formulas were complying with the Infant Formula Act. Indeed, these testing procedures may very well have threatened the availability of infant formula as well as substantially increased infant formula costs. These disadvantages would have resulted in increased risks to consumers as well as placed unnecessary burdens on industry.

The American Medical Association and the American Academy of Pediatrics jointly filed comments with the FDA regarding FDA's proposed quality control procedures. They stated: "If any of this testing redundancy in this detailed scheme of regulation assured that better and 'safer' product would be marketed, even at an increased cost, we would support much of what is proposed. However, we are opposed to this proposal . . ."

The challenge of writing a single set of regulations that encompasses a wide variety of different manufacturing processes is clearly recognized by member companies. Therefore, the Council offered specific suggestions to the FDA regarding feasible alternative regulatory procedures. These recommendations account for the varied manufacturing systems present in industry and, more important, provide assurance that infant formulas comply with the nutrient requirements of the Infant Formula Act.

Infant Formula Council Activities

The Council and its members recognize an obligation to provide factual and authoritative information regarding infant formula and have undertaken a variety of voluntary activities to meet this obligation. One such activity is the Lead Quality Assurance Program. This voluntary program represents the collaboration of all member companies to provide information on the lead content of various infant formulas to the FDA. Results have shown that lead levels have been reduced almost to the detection limits of methodology, an example of member companies' commitment to quality control.

Another service provided by the Infant Formula Council is the development of several manuals which are valuable resources to many facilities. A procedure manual entitled *Methods of Analysis for Infant Formulas* details appropriate methods for microbiological testing, proximate analysis, and vitamin and mineral assays. References are provided. Copies of the manual were provided to both federal and state regulatory agencies when it was first developed in 1973. It currently is in the process of being revised and expanded. Also, a manual to assist laboratories in proper identification of the occasional instance of microbial spoilage was developed and generally distributed to pediatric hospitals and governmental agencies. This manual is also in the process of revision.

A draft copy of a third manual (a physical properties manual) has been developed and sent to FDA. It describes changes that, while not health-threatening, may affect the appearance of infant formula. This manual provides assistance in evaluating the physical stability of liquid formulas and is useful for personnel involved in infant formula inspection programs.

Industry also participated in other projects to improve the safety of infant formula use. One example is the addition of dilution symbols to labels of concentrated liquid infant formulas in response to consumer desires and needs. Based on the results of 2 market research studies, manufacturers now include symbols for adding water on the major display panels of concentrated infant formula products. These symbols were shown to be understandable to a major percentage of the non-English-reading parents to whom they were shown.

The Infant Formula Council also acted promptly in response to an FDA request for information on labeling and export. The Council labeling report to FDA provides an in-depth discussion on infant formula labeling and addresses such topics as the proper use of formula, dilution symbols, standard preparation instructions, color coding, bilingualism, medical information, label endorsement of breast-feeding, and precautions to be taken during the use of the product. The final labeling report submitted to Congress by FDA and the Department of Health and Human Services concluded in part:

> "While there are only limited scientific data, information available indicates that there is no evidence of significant or wide-spread problems attributable to improper or inadequate labeling of infant formulas.
>
> "The infant formula industry has shown an interest in adopting—and a willingness to adapt—to changing needs for the labeling of infant formulas."

Examples of important initiatives voluntarily undertaken by members of the infant formula industry include open dating, pictographic preparation instructions, and the provision of educational materials. We believe the infant formula industry has been highly responsive to the needs of infants, parents, and physicians; we intend to continue and strengthen this responsiveness in the future.

TECHNICAL COMMUNICATIONS

Survival and Movement of *Clostridium perfringens* in Sewage-Treated Soil

WILLIAM D. ST. JOHN and JACK R. MATCHES
University of Washington, Institute for Food Science and Technology, College of Ocean and Fishery Sciences, Seattle, WA 98195

The survival and movement of *Clostridium perfringens* in soil were studied. Fresh dewatered sewage sludge was added to 2 plots of tilled soil. During analyses for 24 weeks, some movement of spores from surface sludge into soil was obtained but little movement through the soil took place. In laboratory studies, columns of soil with a surface layer of sludge were subjected to the equivalence of 67 in. of rain. During this treatment, slow movement through the soil was obtained, but organisms were not detected in column effluents. These data show that *C. perfringens* will survive for at least 24 weeks in soil, with little movement. The application of sewage sludge increases the total numbers of *C. perfringens* in soil, but should have little effect on food poisoning from foods grown in treated soil.

The disposal of sewage in a safe and economical fashion constitutes a major problem facing our expanding urban areas. Sludge constitutes the solid materials that have been separated from liquid sewage. Most sludges considered for disposal have a moisture content of approximately 60% and have been digested by the action of anaerobic bacteria. The sludge at this point contains a 50:50 ratio of organic to inorganic compounds and a thriving population of microorganisms.

One method of disposal considered the most economical for some communities is the use of sewage sludge as landfill. Another use with great potential is as a soil conditioner. One of the principal arguments against the use of sludge on soil is the presence of pathogenic microorganisms, including bacteria, viruses, and parasites (1) which may contaminate crops such as root vegetables which are eaten raw.

Clostridium perfringens was used as the test organism in this study. As a spore former it has the potential for remaining viable in soil for long periods of time. The organism is a common soil inhabitant, has been used as an indicator of fecal contamination, and is present in sewage sludge. The movement of bacteria from sludge applied to soil into the surface streams and ground water has been of some concern.

This study involved 2 main sections: survival and movement of the test organism in soil and sludge, and laboratory studies on the movement of *C. perfringens* in soil.

Materials and Methods

Counting method: *C. perfringens* was detected and counted with a 3-tube MPN (most probable number) method using an iron-milk medium incubated at 45°C (2). *C. perfringens* was detected by the presence of the classical stormy fermentation.

Survival studies: The survival and persistence of *C. perfringens* in soil was determined by adding domestic sewage sludge to soil. Two plots of land (8 × 12 ft each) adjacent to the University of Washington campus were selected because the area had been undisturbed for many years. Both plots were tilled with a garden tiller, and 1 in. of dewatered domestic sludge (collected from Seattle's West Point plant) was added to the surface of the soil. One plot was tilled again to distribute the sludge in the top 6–7 in. of soil. Sludge was left on the surface of the other plot. Soil samples of approximately 100 g each were collected with sterile spatulas from the surface and 3 and 6 in. below the surface for approximately 6 months. The duplicate samples for each test were collected randomly from each 96 sq. ft sampling area. Efforts were made to sample each spot only once. Samples were taken to the laboratory and analyzed immediately.

Ten gram samples of soil were mixed with 90 mL 0.01% peptone water. Subsequent dilutions were prepared using the same diluent. One mL of the appropriate dilutions was added to each of the 3 milk tubes for each dilution.

Movement of organism through soil: Small (2.5 cm diam.) columns were prepared by adding heat-sterilized western Washington soil to a depth of 6 cm. One gram of fresh dewatered

Contribution No. 598, School of Fisheries, University of Washington, Seattle, WA. Work supported by funds made available to the University of Washington by the Public Health Service Biomedical Research Support Grant.
Received November 5, 1981. Accepted March 23, 1982.

0004-5756/82/6506-1514–03$01.00

Table 1. Log numbers[a] of *Clostridium perfringens* recovered from soil with sludge applied on the soil surface

Sampling time after application, weeks	Sampling depth		
	Surface	3 in. below surface	6 in. below surface
1	6.56	4.46	4.15
2	6.05	4.15	4.53
3	6.46	4.45	4.30
4	6.64	4.30	4.15
6	6.43	4.15	4.43
8	6.43	4.15	4.43
12	6.64	4.30	4.30
18	5.43	5.30	5.56
24	5.64	5.62	5.56

[a] Numbers/g soil.

Table 2. Log numbers[a] of *Clostridium perfringens* recovered from soil with sludge applied to the soil surface and tilled into the top 6–7 in. of soil

Sampling time after application, weeks	Sampling depth		
	Surface	3 in. below surface	6 in. below surface
1	5.54	5.30	5.43
2	5.54	5.36	5.59
3	5.64	5.81	5.88
4	5.64	5.64	5.56
6	5.43	5.62	5.56
8	5.64	5.64	5.62
12	5.30	5.64	5.64
18	5.46	5.15	5.15
24	5.43	5.46	5.15

[a] Numbers/g soil.

sewage sludge was added to the top of the semi-packed soil in each of 2 columns. Sterile water was continually added dropwise over a period of 24 h reaching the equivalent of 67 in. of rainfall. The total water added (67 in.) is not significant except this amount is greater than the annual rainfall.

Results

Average *C. perfringens* counts in duplicate samples each collected from the surface and 3 and 6 in. below the surface are shown in Table 1. Soil from the surface contained approximately 2 logs greater *C. perfringens* counts than did soil collected 3 and 6 in. below the surface. The high counts of log 6 on the surface were attributed to the dewatered sludge added. Soil collected below the surface had higher *C. perfringens* counts than were expected and fell into the log 4 range. Samples were collected weekly and very little movement of the organisms took place until after 12 weeks. At the 18-week sampling, the surface counts had decreased by 1 log and the counts of soil collected at depths of 3 and 6 in. had increased by 1 log. No further change took place by the 24-week sampling.

Average numbers of *C. perfringens* recovered from soil with sludge tilled to a depth of 6 to 7 in. are shown in Table 2. Average counts on the surface and at the 2 depths were very similar. During the 24 weeks of sampling, little movement of the organism through the soil could be detected. In addition, survival of the organism was good with very little change during the sampling period.

Although the data in Tables 1 and 2 indicated very little movement of the test organism through soil as a result of rainfall, laboratory studies were designed to measure the movement of *C. perfringens* under controlled conditions. Water was continually added dropwise onto the surface of two 2.5 cm columns packed with soil and layered on the surface with sludge. After the addition of water equivalent to 67 in. of rain (which is in excess of the annual rainfall in the Seattle area), determinations for *C. perfringens* were run. Soil samples collected at depths of 0–2, 2–4, and 4–6 cm and the glass wool plug in the bottom of the columns were assayed for the test organism. A gradient was obtained as shown in Table 3. However, little movement of the organism through soil was obtained even with the equivalence of 67 in. of rain. During the application, no *C. perfringens* was detected in the eluate. The counts at the glass wool plug were log 2.97 and increased to log 6.04 at the surface.

Discussion

The spreading of sewage sludge on the surface of soil and the mixing of this sludge into the soil with a tiller are 2 types of application that could be used on farm land. *Clostridium perfringens* will survive for at least 24 weeks in soil and sludge. Lack of a rapid change in numbers indicated the presence of spores, which was confirmed. Total

Table 3. Log numbers[a] of *Clostridium perfringens* isolated from sludge overlaid with soil in a glass column to which was added the equivalent of 67 in. of water

Sampling depth, cm	No. of *C. perfringens*
0–2	6.04
2–4	5.18
4–6	3.97
Glass wool plug	2.97

[a] Log numbers/g soil.

counts and spore counts determined after heating the soil for 10 min at 60°C were almost identical. The spores which are very resistant to environmental conditions are either attached to soil particles or protected from rapid movement during periods of rainfall. The data do indicate that rainwater washed surface contamination slowly into the soil, perhaps as a result of the beating action of raindrops. The numbers of *C. perfringens* recovered from soil with sludge tilled to a depth of 6–7 in. (Table 2) were approximately the same from surface and subsurface samples. These samples show no movement of the organism with percolation of rainwater falling over a period of 24 weeks. All these studies were carried out during the summer and into the fall and winter months, the rainy season in Seattle, which averages 38–39 in. of rain.

These data are in agreement with other studies which show *Escherichia coli* and *Salmonella* to persist in soil (3–6). Because *C. perfringens* is present in soil in the spore form, it is much more resistant to environmental factors than are vegetative cells and can persist until triggered to germinate by some factor(s).

Because the field studies showed a lack of movement of *C. perfringens* spores in soil, laboratory studies were designed to measure movement under controlled conditions. The movement of organisms through soil in columns substantiated the work of Edmonds (3), who reported that total bacterial populations and fecal bacteria moved from the sludge to the soil beneath but few moved past the first 5 cm. This relieves some of the danger of organisms washing into ground water and streams through the soil during periods of rainfall when sludge is applied to the soil. However, during heavy runoff when soil is removed, the organisms attached to soil particles would also be removed and could contaminate streams.

These data are applicable to the soil type tested. Other soils with different physical and chemical characteristics could behave differently.

Clostridium perfringens has been called the most widely distributed pathogen (7) and may be isolated with ease from almost any environment. This ubiquitous organism is already present in many soil samples (8) and additional numbers will be contributed by the application of sewage sludge. Land used as pasture for large animals may receive large numbers of *C. perfringens* from manure and this has not been generally recognized as a potential health hazard (9). Not all animals shed *C. perfringens* and only 2 of 113 samples of cattle feces contained this organism (10). Yet the contamination of food crops with soil and its microbial flora has been of some concern, and further research is needed to determine the contribution of soil microflora to agricultural products. Many nonsterile foods contain *C. perfringens* (11), presumably as a result of soil or dust contamination. High numbers of cells are required for food poisoning but this pathogen generally becomes a hazard only in foods subjected to poor handling practice such as temperature abuse.

REFERENCES

(1) Pahren, H. R., Lucas, J. B., Ryan, J. A., & Dotson, G. K. (1979) *J. Water Pollut. Control Fed.* **51**, 2588
(2) St. John, W. D., Matches, J. R., & Wekell, M. M. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1129–1133
(3) Edmonds, R. L. (1976) *Appl. Environ. Microbiol.* **32**, 537–546
(4) Hess, E., & Breer, C. (1975) *Zentralbl, Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B* **161**, 54–60
(5) Mallman, W. L., & Litsky, W. (1951) *Am. J. Public Health* **41**, 38–44
(6) Watson, D. C. (1980) *Water Pollut. Control* **29**, 11–17
(7) Smith, L. D. S., & Holdeman, L. V. (1968) *The Pathogenic Anaerobic Bacteria*, C. C. Thomas Co., Fort Lauderdale, FL
(8) Walker, H. W. (1975) *Crit. Rev. Food Sci. Nutr.* (Nov.), 71–104
(9) Hudson, J. A., & Fennel, H. (1980) *Water Pollut. Control* **79**, 370–384
(10) Hobbs, B. C., Smith, M. E., Oakley, C. L., & Warrack, G. H. (1953) *J. Hyg.* **51**, 75–101
(11) Strong, D. H., Canada, J. C., & Griffiths, B. B. (1963) *Appl. Microbiol.* **11**, 42–44

Gas-Liquid Chromatographic Determination of Chlordimeform and Its Metabolites in Cargo Rice and Husk

DE-FANG FAN and SHIN-DING GE
Department of Plant Protection, Section of Pesticide Residues, Agricultural University of Zhejiang, Peoples Republic of China

A specific method is described for the alkali flame ionization gas-liquid chromatographic determination of chlordimeform (*N'*-(4-chloro-o-tolyl)-*N,N*-dimethylformamidine) and 3 potential metabolites in cargo rice and husk. Samples are extracted with absolute alcohol or hexane, and cleaned up on neutral alumina columns. Residues of chlordimeform and its metabolites are chromatographed directly on a column of 1% DEGS coated on 60-80 mesh 405 support (PEG 20M bonded phase). The detection limits for chlordimeform, 4-chloro-o-toluidine, 2,2'-dimethyl-4,4'-dichloroazobenzene, and *N*-formyl-(4-chloro-o-toluidine) are 0.03, 0.028, 0.11, and 0.43 ppm for cargo rice and 0.03, 0.028, 0.22 and 0.43 ppm for husk, respectively.

Chlordimeform, *N'*-(4-chloro-*o*-tolyl)-*N,N*-dimethylformamidine (also named chlorphenamidine) is an acaricide developed by both CIBA Ltd (Galecron) and Schering AG (Fundal) applicable to control of rice insects (1). It is currently being tested in Japan as a systemic insecticide to control rice insects such as paddy stem borer (*Scirpophaga incertulas*), rice stem borer (*Chilo suppressalis*), and leaf folder (*Cnaphalocrocis medinalis*), etc; its safety is also under consideration.

Gas-liquid chromatographic (GLC) methods for the determination of chlorodimeform residues in plants have been described (2–4). These methods are based on the determination of 4-chloro-*o*-toluidine after acid hydrolysis and derivatization to form the iodinated derivative which is measured by electron capture or flame ionization detection. These methods do not allow for direct detection of chlordimeform and its metabolites.

In this report we describe a new method to directly determine residues of chlordimeform and its degradative products in cargo rice and husk by using gas-liquid chromatography and rubidium silicate alkali flame ionization (nitrogen type) detection.

Received May 6, 1981. Accepted June 8, 1982.

METHOD

Reagents

All solvents were reagent grade and distilled in all-glass apparatus.

(a) *Standards.*—Chlordimeform (obtained from West-North University, Department of Chemistry); 4-chloro-*o*-toluidine, *N*-formyl-(4-chloro-*o*-toluidine), and 2,2'-dimethyl-4,4'-dichloroazobenzene (obtained from Chemical Industrial Research Institute of Zhejiang Province).

(b) *Neutral aluminum oxide.*—Chromatographic grade, 100–200 mesh. Activate 2 h at 130°C and let cool in tightly stoppered bottle; deactivate by adding 5% water before use.

Apparatus

(a) *Gas-liquid chromatograph.*—Model 102G (Shanghai Analytical Apparatus Factory, People's Republic of China). Operating conditions—Temperatures (°C): column 164 (for chlordimeform and 4-chloro-*o*-toluidine) and 220 (for *N*-formyl-(4-chloro-*o*-toluidine) and 2,2'-dimethyl-4,4'-dichloroazobenzene); detector 270; injection port 240. Attenuation 1/8 × 1000; nitrogen flow (99.99%) 17.5 mL/min (for chlordimeform and 4-chloro-*o*-toluidine) and 20 mL/min (for *N*-formyl-(4-chloro-*o*-toluidine) and 2,2'-dimethyl-4,4'-dichloroazobenzene); air flow 95 mL/min; hydrogen flow 14.4 mL/min. Steel column: 100 cm × 2 mm id, packed with 1% DEGS on 60–80 mesh 405 support (PEG 20M bonded phase), which is prepared by Talien Chemistry-Physics Research Institute. Rubidium silicate heater conditions: electric current 40 amp.; voltage 0.87 V; polarizing voltage −180 V.

(b) *Kuderna-Danish (K-D) evaporative concentrator.*

(c) *Chromatographic column.*—Glass, 20 cm × 2 cm packed with 15 g deactivated alumina and overlaid with ca 5 g anhydrous Na_2SO_4.

Preparation of Sample

(The dehusking machine divides the grain samples into cargo rice and husk.) Extract 10 g

0004-5756/82/6506-1517-04$01.00

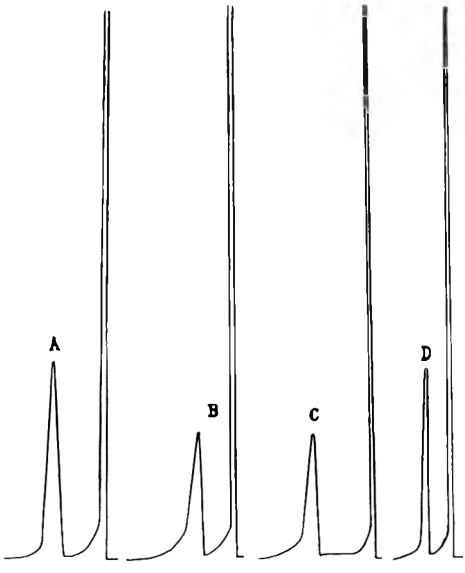


Figure 1. Gas chromatograms of chlordimeform and its metabolites under described operating conditions: A, 2,2′-dimethyl-4,4′-dichloroazobenzene (t_r 1.3 min); B, *N*-formyl-(4-chloro-*o*-toluidine) (t_r 0.85 min); C, chlordimeform (t_r 1.4 min); D, 4-chloro-o-toluidine (t_r 0.62 min).

ground sample of cargo rice or husk extract for 8 h in Soxhlet with 70 mL absolute alcohol (for detection of chlordimeform, *N*-formyl-(4-chloro-*o*-toluidine), and 4-chloro-*o*-toluidine) or hexane (for 2,2′-dimethyl-4,4′-dichloroazobenzene). Transfer filtrate into K-D evaporative concentrator; wash extract residue twice with 100 mL of same solvent. (The *p*-values of chlordimeform, *N*-formyl-(4-chloro-*o*-toluidine), 4-chloro-*o*-toluidine, and 2,2′-dimethyl-4,4′-dichloroazobenzene in these solvents are 0.29, 0.02, 0.19, and 1, respectively.)

Column Cleanup and Determination

Combine all filtered solutions, evaporate solvents by means of K-D concentrator under vacuum, and concentrate to ca 5 mL. Transfer concentrated solution onto deactivated alumina column and elute with absolute alcohol (for polar compounds) or hexane (for nonpolar compound). Collect alcohol eluate from 10 to 40 mL for determination of chlordimeform, 4-chloro-*o*-toluidine, and *N*-formyl-(4-chloro-*o*-toluidine); collect hexane eluant to 100 mL for determination of 2,2′-dimethyl-4,4′-dichloroazobenzene. Concentrate eluates to appropriate volume and inject 1–5 μL into gas chromatograph.

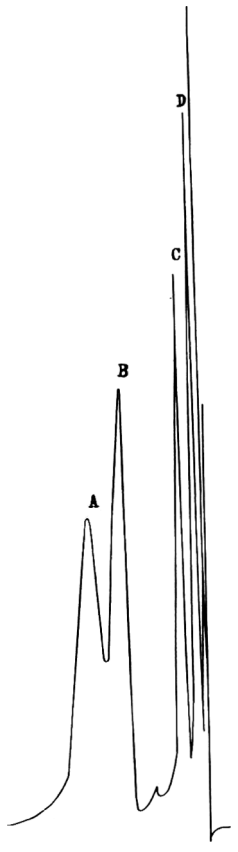


Figure 2. Chromatogram of mixed solution of chlordimeform and its metabolites under alternative operating conditions: column 200°C; injection port 260°C; nitrogen flow 17 mL/min; air flow 92 mL/min; hydrogen flow 10 mL/min; attenuation 1/8 × 1000. A, 2,2′-dimethyl-4,4′-dichloroazobenzene (t_r 3.3 min); B, *N*-formyl-(4-chloro-o-toluidine) (t_r 2.2 min); C, chlordimeform (t_r 0.53 min); D, 4-chloro-o-toluidine (t_r 0.38 min).

Results and Discussion

Suitability and Sensitivity

The method described allows detection of the parent compound and its metabolites directly on the same 1% DEGS column in one determination. Individual chromatograms are shown in Figure 1. The retention times of 2,2′-dimethyl-4,4′-dichloroazobenzene, *N*-formyl-(4-chloro-*o*-toluidine), chlordimeform, and 4-chloro-*o*-toluidine were 1.3, 0.85, 1.4, and 0.62 min, respectively.

Under different conditions, a 5 μL solution containing all 4 compounds was injected and analyzed. The chromatogram is shown in Figure 2.

Table 1. Residues of chlordimeform and metabolites in field-treated rice (1979)

Applied method	Doses of spraying active ingred., g/ha	Times of spraying	Days after spraying	Chlordimeform, ppm		4-Chloro-*o*-toluidine, ppm		*N*-formyl-(4-chloro-o-toluidine, ppm		2,2'-Dimethyl 4,4'-dichloroazobenzene, ppm	
				Cargo rice	Husk	Cargo rice	Husk	Cargo rice	Husk	Cargo rice	Husk
Early Rice											
Spraying	289	1	38	ND[a]	0.24–0.28	ND[b]	ND[b]			ND[c]	ND[d]
	750	1	25	ND	0.95	ND	ND	ND[e]		ND	ND
	375	3	28	ND	0.52–1.1	ND	ND	ND[e]	1.1–3.6	ND	ND
Applied into the soil[f]	1500	1	20	ND	1.0	ND	ND			ND	ND
		1	25	ND	0.23	ND	ND			ND	ND
		1	30	ND	0.16	ND	ND			ND	ND
		1	≥35	ND	trace[g]	ND	ND			ND	ND
		1	≥55	ND	ND[a]	ND	ND			ND	ND
Late Rice											
Spraying	375	3	25	ND	0.40–0.49	ND	ND			ND	ND
Applied into the soil[f]	1500	1	61	ND	trace[g]	ND	ND			ND	ND

[a] <0.03 ppm.
[b] <0.02 ppm
[c] <0.11 ppm
[d] <0.22 ppm
[e] <0.43 ppm
[f] Done by root-zone application using apparatus 6 cm depth under soil surface.
[g] About 0.03 ppm.

The minimum detectable quantities were 0.614 ng, 0.284 ng, 0.85 ng, and 2.24 ng for chlordimeform, 4-chloro-*o*-toluidine, *N*-formyl-(4-chloro-*o*-toluidine), and 2,2′-dimethyl-4,4′-dichloroazobenzene, respectively. The standard response curves for the 4 compounds were linear in the range of 4.18–418 ng, 0.35–350 ng, 18–5400 ng, and 14–840 ng, respectively.

Analysis of Field Samples

The detection limits for 10 g cargo rice or husk are as follows: chlordimeform 0.03 ppm, 4-chloro-*o*-toluidine 0.028 ppm, *N*-formyl-(4-chloro-*o*-toluidine) 0.43 ppm, 2,2′-dimethyl-4,4′-dichloroazobenzene 0.22 ppm (in 10 g husk) and 0.11 ppm (in 20 g cargo rice).

When 8.3–41.8 μg chlorodimeform was added to 10 g background samples, recoveries were 81–93% for cargo rice and 103–104% for husk; 35 μg 4-chloro-*o*-toluidine added to 10 g background samples was recovered at 71–73% for both cargo rice and husk; 2.7–276 μg 2,2′-dimethyl-4,4′-dichloroazobenzene added to 20 g cargo rice was recovered at 81.8–112% and 9.74–97.4 μg 2,2′-dimethyl-4,4′-dichloroazobenzene added to 20 g husk was recovered at 109–118%; 68 μg *N*-formyl-(4-chloro-*o*-toluidine) added to 10 g husk was recovered at 66%.

Analytical data from a field application experiment conducted in 1979 are shown in Table 1. Residues of chlordimeform and its metabolites were not found in either early or late cargo rice, but were found in the husk. No residues of 2,2′-dimethyl-4,4′-dichloroazobenzene or 4-chloro-*o*-toluidine were found in the husk. The only metabolite determined was *N*-formyl-(4-chloro-*o*-toluidine) found in the husk of the rice treated with a high dose (375 g/ha) or replicated spray (3 times).

Acknowledgments

The authors thank Zhang Le-feng and Wang Xue-tao, Chemistry-Physics Research Institute of Talien, and Zhu Ke-ming, Section of Pesticide Residues, Agricultural University of Zhejiang, for their cooperation on the use of the alkali flame ionization detector. We also thank Qu Tian-Jiang for his interest in our work.

REFERENCES

(1) Hirano, T., Kawasak, H., Sninohara, H., & Kitagaki, T. (1972) *Botyu-Kagaku* **37**, 137–141
(2) Kossmann, K., Geissbuhler, H., & Boyd, V. F. (1971) *J. Agric. Food Chem.* **19**, 360–364
(3) Geissbuhler, H., Kossmann, K., & Boyd, V. F. (1971) *J. Agric. Food Chem.* **19**, 365–371
(4) Su, G. C. C., & Zabik, M. J. (1972) *J. Agric. Food Chem.* **20**, 320–323

Limitations in Protection Afforded by Gloves in Laboratory Handling of Aflatoxins

MARCEL CASTEGNARO, HANS P. VAN EGMOND[1], WALTER E. PAULSCH[1], and JOCELYNE MICHELON
International Agency for Research on Cancer, 150 Cours Albert Thomas, 69372 Lyon Cédex 2, France

The permeability of latex and vinyl laboratory gloves by aflatoxins in chloroform or dimethyl sulfoxide (DMSO) has been investigated. Latex gloves provide good protection against permeation by aflatoxins in DMSO, but aflatoxins in chloroform permeate all types of laboratory gloves. If gloves are contaminated when aflatoxins in chloroform are handled, an immediate change of gloves is recommended.

The use of rubber or vinyl gloves is good laboratory practice when working with chemical carcinogens. It has been demonstrated, however, that this generally accepted safety procedure sometimes has limitations in its effectiveness, as for nitrosamines dissolved in halogenated solvents (1–3). Some other carcinogens may permeate laboratory gloves, depending on the chemical structure of the compound(s) involved, their solubility in the solvent used, and the resistance of the glove to this solvent.

Aflatoxins, a well known group of chemical carcinogens (4, 5), are often handled in solutions of chloroform, because this solvent is frequently specified in extraction procedures, and in di-

[1] National Institute of Public Health, Postbox 1, 3720 BA Bilthoven, The Netherlands.
Received July 29, 1981. Accepted April 14, 1982.

0004-5756/82/6506-1520-04$01.00

Table 1. Effect of chloroform and dimethylsulfoxide (DMSO) on different types of laboratory gloves and permeation of gloves by aflatoxin B_1

Type of glove	Chloroform	DMSO
Thin latex	strong swelling mechanical weakening transfer of aflatoxin	no swelling no mechanical weakening no transfer of aflatoxin
Thick latex	strong swelling mechanical weakening transfer of aflatoxin	no swelling no mechanical weakening no transfer of aflatoxin
Vinyl	moderate swelling mechanical weakening transfer of aflatoxin	moderate swelling mechanical weakening transfer of aflatoxin

methyl sulfoxide (DMSO), which is often used as a vehicle in administering aflatoxin to experimental animals. This prompted us to investigate the effectiveness of various types of gloves as a boundary protection against permeation by aflatoxin from chloroform and DMSO solutions. It should be noted that the experiments were not designed as a complete study of all possibilities of permeation.

Experimental

Thin latex gloves (0.1–0.14 mm), thick latex gloves (0.5–0.8 mm), and vinyl gloves (0.8–1 mm) from 7 European and American manufacturers were investigated in 2 series of experiments.

In the first series, the diffusion of aflatoxin B_1 was investigated for solutions of chloroform or DMSO: Ten mL of a solution containing 1 mg B_1/L was transferred into the finger of a glove, the same volume of solvent was placed in a small beaker, and the glove finger was immersed in the beaker so that liquid levels were nearly equal inside and outside the glove finger. At regular intervals, the outer solution was stirred and small amounts of solvent were taken for thin layer chromatographic (TLC) analysis for the presence of aflatoxin.

The second series of experiments took into consideration the tendency of perspiration to accumulate rapidly on the inside glove surface in contact with the skin. Therefore, the resistance of thin latex and vinyl gloves against permeation into saline by aflatoxins dissolved in chloroform was studied. Ten mL 4% saline solution was transferred into the finger of a latex glove that was immersed in a beaker containing 10 mL of a chloroform solution containing 2 mg/L of each of aflatoxins B_1, B_2, G_1, and G_2.

Table 2. Permeation of aflatoxins B_1, B_2, G_1, and G_2 from chloroform solution into 4% saline solution

Aflatoxin[a]	Approx. amt (ng) in saline soln	Amt transferred, ‰
B_1	5	0.5
B_2	5–10	0.5–1
G_1	15–20	1.5–2
G_2	20	2

[a] 10^4 ng of each aflatoxin/10 mL $CHCl_3$.

After 15 min, the saline solution was analyzed by TLC for aflatoxins. The reverse situation was also studied: Ten mL of a solution of 1 mg B_1/L chloroform was added to the finger of a vinyl glove that was then placed in a beaker containing 4% saline solution. In both experiments, chloroform transpired through the gloves. Only in the case of vinyl gloves were drops of chloroform visible in the saline. This transpired chloroform was analyzed at regular intervals. Transpiration of DMSO through vinyl gloves was also studied.

In order to find the theoretical maximum transfer of aflatoxin B_1 from chloroform into saline solution, the partition of B_1 between these phases (without any boundary) was determined by shaking a solution of 1 mg B_1/L chloroform with an aliquot of 4% saline solution. The saline was re-extracted with chloroform and this extract was analyzed by TLC.

For TLC analysis, chloroform solutions were directly concentrated before spotting; saline solutions were extracted with chloroform and the extract was concentrated. DMSO solutions were mixed with an equal volume of dichloromethane and evaporated to dryness, using a rotary evaporator under reduced pressure. The extracts were spotted on Kieselgel 60 TLC plates and the plates were developed in chloroform–acetone (9 + 1).

Results and Discussion

Table 1 presents the qualitative aspects of the effect of chloroform and DMSO on different types of gloves and of the transfer of aflatoxins through these gloves.

Figure 1 summarizes the quantitative aspects of B_1 permeation of the gloves. All types tested allowed permeation by B_1 in chloroform solutions. For latex gloves, the transfer depends on time and the thickness of the material; thick gloves allowed less permeation by B_1 than did thin surgical gloves. There was no permeation of latex gloves by B_1 in DMSO solutions; how-

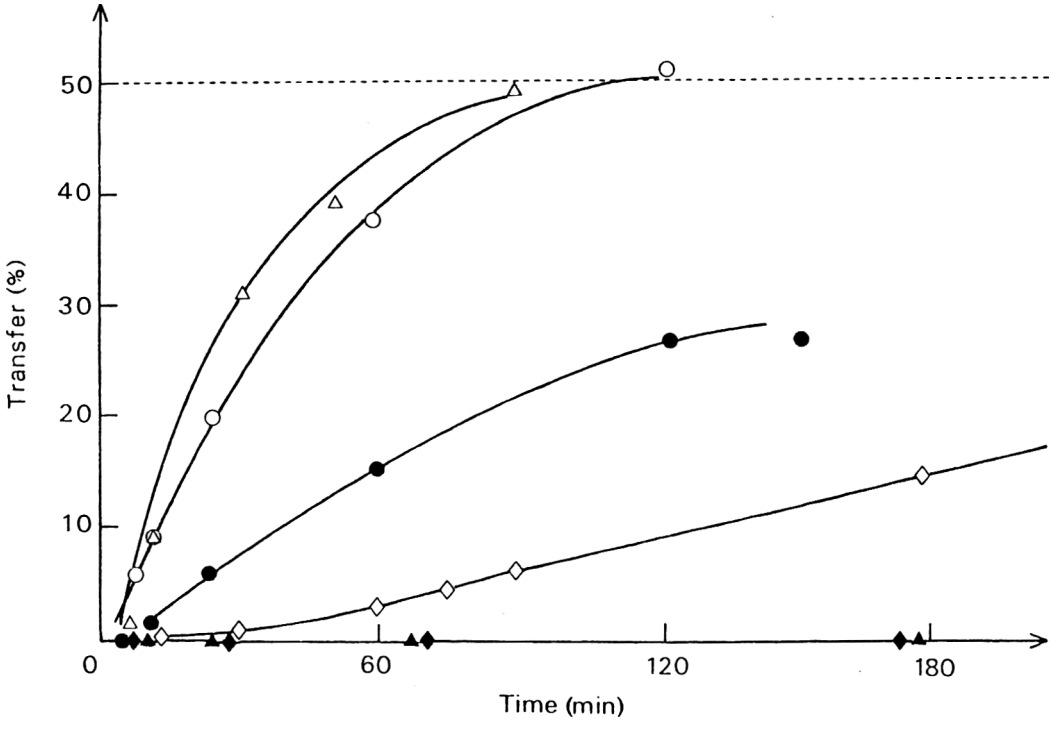


Figure 1. Permeation of aflatoxin B_1 in chloroform and DMSO solutions (same inside and outside the glove) through latex and vinyl gloves as a function of time: △, $CHCl_3$ solution, thin latex; ▲, DMSO solution, thin latex; ◊, $CHCl_3$ solution, thick latex; ♦, DMSO solution, thick latex; ○, $CHCl_3$ solution, vinyl; ●, DMSO solution, vinyl.

ever, vinyl gloves permitted transfer of B_1. Results in Figure 1 and Table 1 show that permeation by B_1 correlates with the mechanical strength and swelling of the glove material in the solvent concerned.

Aflatoxin permeation of thin latex gloves from chloroform into saline was about 0.5‰ for B_1 and somewhat higher for the more polar aflatoxins (Table 2). This approaches the theoretical maximum amount that would permeate from chloroform into 4% saline solution, found to be about 0.8‰ for B_1.

Although not shown in Table 2, we found that for thick latex gloves the theoretical maximum amount is also approached; however, it takes much more time to reach equilibrium. Transpiration of chloroform and of DMSO was observed through vinyl gloves; slight transpiration of chloroform was noted for thin latex gloves. Analysis of the drops of chloroform that transpired through vinyl gloves into the saline showed a slight increase of B_1 concentration with time (Figure 2), until the concentrations of B_1 in chloroform, inside and outside the glove, have become equal. Filter action of the glove for B_1 might explain this increase, which diminishes when the mechanical strength of the glove decreases. Another possibility could be specific absorption of B_1, which occurs until equilibrium is reached. Neither transpiration of DMSO nor permeation by aflatoxins from DMSO solutions through thin latex was investigated, because it was evident from the first series of experiments that aflatoxins dissolved in DMSO did not permeate latex gloves.

Conclusions

The experiments demonstrated that the use of latex and vinyl laboratory gloves does not always fully protect the user when solutions of aflatoxins in chloroform or DMSO are handled. This is most important when accidental spills occur. Although, in practice, transfer of aflatoxins through the glove seems to be very limited; it depends on time and on the mechanical weakening of the glove by organic solvents, which increases the risk of contact of the skin by aflatoxin-containing solutions. From Table 1, the

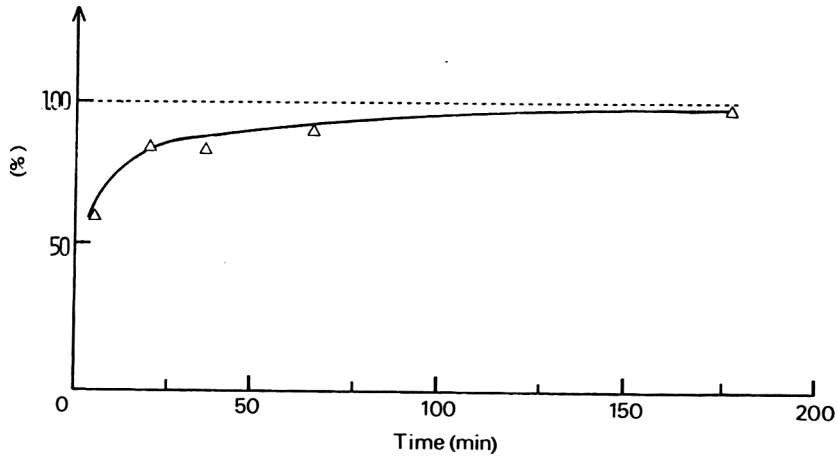


Figure 2. Concentration of aflatoxin B_1 in chloroform drops, transpired through vinyl glove into 4% saline solution as function of permeation time (expressed as percent of original concentration).

conclusion can be drawn that for safe handling of solutions of aflatoxins in DMSO, latex gloves are preferable to vinyl gloves. When chloroform solutions are handled, latex gloves are preferable, because chloroform tends to permeate vinyl gloves, eventually carrying aflatoxin through what appears to be an absorptive barrier (see Figure 2). The thicker the barrier (thick latex, a double pair of thin latex gloves, or the combination of thin latex and vinyl gloves), the better the protection. Therefore, if feasible, one of these possibilities should be used. However, if tactile sense is required to carry out laboratory tasks and therefore thin gloves must be used, thin latex gloves are preferable to vinyl gloves.

Finally, it is strongly recommended that contact between gloves and solutions containing aflatoxins be kept as short as possible and that laboratory gloves not be re-used after they have come into contact with solutions containing aflatoxins.

Acknowledgments

The authors thank P. L. Schuller for his helpful comments and suggestions in preparing this manuscript.

References

(1) Sansone, E. B., & Tewari, Y. B. (1978) in *Environmental Aspects of N-Nitroso Compounds*, E. A. Walker, M. Castegnaro, L. Griciute, & R. E. Lyle (Eds), International Agency for Research on Cancer, IARC Sci. Publ. No. 19, Lyon, France, pp. 517–529

(2) Gough, T. A., Webb, K. S., & McPhail, M. F. (1978) in *Environmental Aspects of N-Nitroso Compounds*, E. A. Walker, M. Castegnaro, L. Griciute, & R. E. Lyle (Eds), International Agency for Research on Cancer, IARC Sci. Publ. No. 19, Lyon, France, pp. 531–534

(3) Walker, E. A., Castegnaro, M., Garren, L., & Pignatelli, B. (1978) in *Environmental Aspects of N-Nitroso Compounds*, E. A. Walker, M. Castegnaro, L. Griciute, & R. E. Lyle (Eds), International Agency for Research on Cancer, IARC Sci. Publ. No. 19, Lyon, France, pp. 535–543

(4) Purchase, I. F. H. (Ed.) (1974) *Mycotoxins*, Elsevier Scientific Publishing Co., Amsterdam, The Netherlands

(5) International Agency for Research on Cancer (1976) *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man*, Vol. 10, Lyon, France, pp. 51–72

FOR YOUR INFORMATION

AOAC Scholarship Award for 1982 to go to Carol Lasko of Dunsmuir, CA

Carol Lasko, an outstanding student at the College of the Siskiyous, Weed, California, is the winner of a two-year, $1000 scholarship sponsored by AOAC.

Ms. Lasko has shown unusual ability in chemistry, especially analytical chemistry. She is enrolled in the college's work-study program. As laboratory assistant to the chairman of the college's Natural and Applied Science Division, mostly on her own initiative and with minimal direction and assistance, she researched, tested methods, and compiled results on a project on alternatives to hydrogen sulfide separations for qualitative analysis.

Ms. Lasko's superior performance at the College of the Siskiyous is a continuation of the straight A record she earned at the City College of San Francisco in 1968 and 1969. She hopes to continue her studies at Southern Oregon College and eventually to earn her graduate degree.

Each year AOAC awards a two-year scholarship to a college sophomore who is studying a subject important to public health and agriculture. To qualify, the student must be in need of financial aid, maintain at least a B average during the first two years of undergraduate study, and plan to do research, regulatory work, quality control, or teach in an area of interest to the AOAC.

Nominations for the 1983 award must be received before 1 May 1983. Send six copies of a nomination letter and two supporting reference letters to AOAC, 1111 N 19th St, Arlington, VA 22209.

AOAC to Hold Eighth Annual Spring Workshop and Exposition, April 19-21, 1983

The Association of Official Analytical Chemists (AOAC) will sponsor the 8th Annual Spring Workshop and Exposition April 19-21, 1983 at the Sheraton West Hotel, Indianapolis, IN. The sessions are planned to provide state-of-the-art presentations on the following subjects: sampling, chromatographic and spectroscopic techniques, mycotoxins, trace metals, pesticide formulations, monitoring for organic residues, problems associated with instrumentation and automation, new instrumentation, antibiotic analysis in foods and feeds, vitamin analysis, pharmaceuticals, high pressure liquid chromatography, drug residues in foods and feeds, toxicology, forensics, veterinary toxicology, microbiological methods, microbiology of waste waters, food microbiology, and medical devices.

An exposition of analytical instruments, apparatus, supplies, and services will be held in conjunction with the conference.

Registration fees are as follows: pre-registration fee $60 (deadline is April 4, 1983), on-site registration $75, student registration $20, and registration by the day $35. Registration fee includes 2 lunches plus a wine and cheese party. For room registrations, contact the Sheraton West Hotel, 2544 Executive Dr., Indianapolis, IN 46241 (317/248-2481); please mention the AOAC meeting. Costs for single and double occupancy are $54.60 and $64.00, respectively.

For information contact conference chairman, Lawrence Sullivan, Indiana State Board of Health, 1330 West Michigan St., Indianapolis, IN 46206 (317/633-0224).

Meetings

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 University, 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 and Fats, Chemistry Dept, University of Auckland, Private Bag, Auckland, NZ.

April 7-8, 1983: Committee E-11 on Statistical Methods, ASTM Headquarters, Philadelphia, PA. Contact: Bill Hulse, 215/299-5507.

April 19-21, 1983: AOAC 8th Annual Spring Workshop and Exposition, Sheraton West Hotel, Indianapolis, IN. For information, see article above.

May 24-26, 1983: 37th Annual Quality Congress and Exposition, Boston, MA.

0004-5756/82/6506-1524-02$01.00

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, 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: C. J. Mussinan, International Flavor and Fragrances, Inc., Research and Development, 1515 Highway 36, Union Beach, NJ 07735; 201/264-4500.

Three New Private Sustaining Members Join AOAC

AOAC extends a welcome to the following three new Private Sustaining Members: Boehringer Mannheim Biochemicals, Indianapolis, IN; Campbell Institute of Research and Technology, Camden, NJ; and Norwich Eaton Pharmaceuticals, Inc., Norwich, NY.

NEW PUBLICATIONS

WHO Expert Committee on Biological Standardization. 32nd Report. World Health Organization Technical Report Series, No. 673, 1982. Available from WHO Publications Centre USA, 49 Sheridan Ave, Albany, NY 12210. 180 pp. Price: Sw. fr. 13.-. Arabic, French, and Spanish editions in preparation. ISBN 92-4-120673-X.

The WHO Expert Committee's continuing work is described in this report. Among matters discussed are the following: provision of international standards, establishment of biologicals laboratories, provision of working standards, reconstitution and use of standards, assignment of units of activity, biological standards for clinical chemistry, antibiotic susceptibility tests, poliomyelitis vaccine, requirements for Rift Valley fever vaccine, stability of measles vaccine, test for pyrogens, and biologicals prepared by new technology (DNA recombination and other methods). The Committee also noted the results of studies on certain antibiotics, antigens, blood products and related substances, endocrinological and related substances, and reference reagents.

Adding Nutrients to Foods: Where Do We Go From Here? Edited by James L. Vetter. Published by the American Association of Cereal Chemists (AACC), 3340 Pilot Knob Rd, St. Paul MN 55121, 1982. 152 pp. Price: $18.00 AACC members, $20.00 nonmembers. ISBN 0-913250-25-2.

Based on presentations made at the AACC Workshop on Fortification, January 1982, Arlington, VA, this book analyzes the issue of nutrient fortification on both the domestic and international levels. It covers past and present roles of nutrients in our diets and explores future implications for nutrient addition programs. Contents include current policies and attitudes of government toward nutrient additions, determining the need for nutrient additions, and factors to be considered in implementing nutrient addition programs.

Advances in Cereal Science Technology, Volume V. Edited by Y. Pomeranz. Published by the American Association of Cereal Chemists, 3340 Pilot Knob Rd, St. Paul, MN 55121, 1982. 308 pp. Price: $32.50 AACC members, $36.00 nonmembers. ISBN 0-913250-28-7.

This book compares the merits of increased yields with technological improvement and plant breeding, and highlights new insights being discovered in these areas. It also brings together new information on wheat genetics and enzymes, oats, buckwheat, use potential of rapeseed, and gel electrophoresis of grain proteins.

Contents include chromosomal locations of genes that control wheat endosperm proteins; oats; enzymes in wheat, flour, and bread; buckwheat: description, breeding, production, and utilization; identification of cereal varieties by gel electrophoresis of the grain proteins; and rapeseed.

Physics and Chemistry of the Shroud of Turin. By L. A. Schwalbe and R. N. Rogers. Published by Elsevier Scientific Publishing Co., PO Box 330, 1000 AH Amsterdam, The Netherlands, 1982. Also available from Elsevier North-Holland Inc., 52 Vanderbilt Ave, New York, NY 10017. 49 pp. Price: $11.75.

This publication is a reprint from *Analytica Chimica Acta* (1982, Vol. 135, No. 1, pp. 3–49) of a summary of the 1978 investigation of the shroud of Turin at the Los Alamos National Laboratory, Los Alamos, NM, USA.

Ion-Selective Electrodes 3. Analytical Chemistry Symposia Series, Volume 8. By E. Pungor and I. Buzás. Published by Elsevier Science Publishers, P.O. Box 211, 1000 AE Amsterdam, The Netherlands, 1981. Also available from Elsevier Science Publishing Co., Inc., 52 Vanderbilt Ave, New York, NY 10017. 428 pp. Price: US$100.00; Dfl. 215. ISBN 0-444-99714-8.

The 4 plenary lectures, 5 keynote lectures, and 23 discussion lectures which formed the proceedings of the Third Symposium on Ion-selective Electrodes held at Mátrafüred are collected in this volume. The latest research

results in the field are summarized and discussed. A survey on the aims and tasks of future research activity is included, with emphasis on the problem of standardizing ion-selective electrodes.

Analytica Chimica Acta – Cumulative Index – Volume 1-100. Published by Elsevier Scientific Publishing Co., PO Box 211, 1000 AE Amsterdam, The Netherlands, 1982. 634 pp. Price: US$105.60; 264 Dutch guilders.

A cumulative author, subject, and techniques index for Volumes 1–100 of *Analytica Chimica Acta* published between 1947 and 1978 constitutes this book.

BOOK REVIEWS

Advances in Cereal Science Technology. Volume IV. Edited by Y. Pomeranz. Published by the American Association of Cereal Chemists, Inc., 3340 Pilot Knob Rd, St. Paul, MN 55121, 1981. v + 342 pp. Price: $36.00. ISBN 0-913250-21-X.

Just as demonstrated in previous volumes of this series, the editor fulfills his promise to present balanced and multidisciplinary reviews by scientists from all over the world.

Volume IV consists of five chapters. The first chapter contains a brief account of yeast in modern history, a topic of interest to high school teachers and regulatory chemists. A thorough review of recent biochemical and histochemical studies on the morphology and ultrastructure of the developing wheat endosperm is provided in the second chapter. Sorghum and millet, from biochemistry to food uses, are covered in Chapter 3. Engineering and chemical aspects of high-temperature, short-time extrusion cooking are discussed in Chapter 4 by authors from Finland and France. The last chapter summarizes current knowledge about grain dust, such as risk analysis and prevention of dust explosion. The book contains several references of use to analysts.

JAMES F. LIN

Food and Drug Administration
Division of Food Technology
Washington, DC 20204

Mutagenicity Testing and Related Analytical Techniques. Current Topics in Environmental and Toxicological Chemistry. Volume 3. Edited by R. W. Frei and U. A. Th. Brinkman. Published by Gordon and Breach Science Publishers, Inc., One Park Ave, New York, NY 10016, 1981. 320 pp. Price: $46.50. ISBN 0-677-16300-2.

Current Topics in Environmental and Toxicological Chemistry is a series based on papers from the *International Journal of Environmental Analytical Chemistry* and the *Journal of Toxicological and Environmental Chemistry*. This volume contains selected papers presented at the 10th Annual Symposium on the Analytical Chemistry of Pollutants held in Dortmund, Federal Republic of Germany, May 28–30, 1980.

The papers can be grouped under 5 topics: properties of environmental pollutants to be investigated, criteria for various toxicity tests, description of some acute and subacute toxicity tests, mutation tests, and analytical procedures for environmental pollutants. The book is well organized. The first chapter considers the basic aspects of testing chemicals. The second chapter discusses ecotoxicological tests and stresses substances produced in large quantities and substances whose properties indicate considerable ecotoxicological danger. The author of this chapter discusses different approaches for assessing effects of chemicals in the environment, such as toxicological assays with different species, ecotoxicological assays, and epidemiological assays.

Four chapters of the volume are devoted to carcinomutagen tests—tests which present several problems. First, there is the question of how the huge number of bioassays for carcinogenicity described, using cells, tissues, organs, body fluids, eukaryotes, prokaryotes, etc., with a wide variety of biological end points, contribute to the understanding and prevention of human carcinogenesis. When rodent bioassays for carcinogenicity present problems when extrapolated to humans, how valuable is extrapolation from simpler prokaryotes and non-mammalian eukaryotes? There is also a question as to the significance of the results of these tests. On one hand, mutation tests—essentially crude spot tests—will often, with further improvements, give more meaningful information on mutagenicity than do cancer bioassays. On the other hand, as discussed in this book, the significance and possible hazard of low concentrations of genotoxicants, such as in drinking water, is still to be determined.

One of the more interesting chapters is a discussion of the use of testicular DNA synthesis inhibition as an in vivo system for the detection of carcinomutagens. It is postulated that the DSI, the Ames, and the Hamster Embryo Cell tests can provide a 100% correlation between test result and

carcinogenicity. If such a correlation were reached, one would still be confronted with questions regarding extrapolation to humans.

Eleven chapters consider instrumentation and techniques useful in the analysis of various environmental genotoxicants and cofactors. These include analysis of aromatic amines, PCBs, carbonyl compounds, phenols, and single fibers. In addition, two-photon excitation spectra of PAH, and the direct coupling of a gas-phase enrichment column with a liquid chromatograph are discussed.

This can be a useful volume—an attempt to fuse carcinomutagen bioassay and environmental pollutant analytical procedures. The papers present in-depth reviews and novel procedures, both of considerable interest to a wide range of environmental analysts concerned with genotoxic bioassays and organic analysis.

EUGENE SAWICKI
Center for Environmental Genotoxic Studies
Raleigh, NC 27612

Food Composition and Nutrition Tables 1981-1982. By S. W. Souci, W. Fachmann, and H. Kraut. Published by Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, GFR, 1981. 1352 pp. Price: DM 96.-. ISBN 3-8047-0617-7.

The updated data on generic foods which make up this book are arranged in 17 major food groups. The book is written in English, German, and French with scientific Latin names used in some areas. Each food is first described in calories and amounts of protein, fat, and carbohydrates per serving. The individual constituents, average values for 100 grams of "edible portion," as well as for 100 grams of raw product are given utilizing the dimensions gram, milligram, or microgram. Specific minerals, vitamins, amino acids, and fats contained in the food are also shown. In applicable areas, the crude fiber content is presented.

To avoid variables that may arise for foods such as potatoes which are not readily digestible in the raw state, the values given are for the prepared state of foods as they would ordinarily be consumed.

This book is an excellent reference for scientists and researchers in the area of nutrition, especially for the lesser known nutrients (Mn, Cu, Zn, Cl, F, I, Mo, B, Se, Co, Cr, carotene, vitamin D, vitamin K, biotin, vitamin B_6, pantothenic acid, folacin, and vitamin B-12). In addition, data for many lesser known foods are presented.

Although the overall material is very thorough, there are certain deficiencies that may be of concern to scientists using the book. There are no references for the data which is said to be derived from previous publications, there is no mention of the method of preparation of foods that may be eaten raw or cooked, and the foods are not listed alphabetically in the book except in the index. The few minor shortcomings that are noted, however, do not take away from the overall quality of the material presented.

In summary, this is an excellent reference book on nutrient values in generic foods, including lesser known constituents of foods and many foods that are new to the market.

DANA F. FLAVIN
JEAN A. T. PENNINGTON
Food and Drug Administration
Bureau of Foods
Washington, DC 20204

Pharmaceutical Analyses: Modern Methods, Part A, Drugs and Pharmaceutical Sciences, Volume 11, Edited by James W. Munson. Published by Marcel Dekker, Inc., New York and Basel, 1981. 485 pp. Price: $55.00. ISBN 0-8247-1502-0.

Overall, this volume accomplishes the purpose stated in the editor's preface. It is a bridge between detailed exposition and basic text. Six topics are covered by nine authors. The topics are gas chromatography, pyrolysis—gas chromatography, gas chromatography—mass spectrometry, fluorescence and phosphorescence spectroscopy, liquid scintillation counting, and radio immuno-assay.

It is obvious that the editor exercised a great deal of control and established criteria for the chapters which are, in general, similarly structured. Each gives theory, necessary instrumentation, problems, and applications. Each chapter has an extensive bibliography ranging from 94 references for liquid scintillation to 338 for gas chromatography. The references are as current as can be expected in a bound volume.

There are two indexes, one for drugs and one for authors. These should be useful when

the researcher is looking for applications. The three chapters relating to gas chromatography have some repetition, but not as much as is generally found in a multiple-author tome. The book should be useful to graduate students or researchers looking for ideas and approaches to the analysis of pharmaceuticals, and to regulatory analysts.

EVELYN SARNOFF

Food and Drug Administration
New York District Laboratory
Brooklyn, NY 11232

Maintaining and Trouble-Shooting HPLC Systems – A User's Guide. By Dennis J. Runser. Published by John Wiley and Sons, New York, NY, 1981. xiii + 163 pp. Price: $27.50. ISBN 0-471-06479-3.

The purpose of this book, as stated by the author, is to provide the HPLC user with a comprehensive manual for maintaining and trouble-shooting HPLC systems. This purpose is fulfilled by treating each individual component of the system and with emphasis on preventive maintenance.

One chapter is devoted to the mobile phase, its purity, preparation, compatibility with the HPLC system, and effect of impurities; another chapter covers various solvent delivery systems and states that the selection of a delivery system should include consideration of maintenance, trouble-shooting ease, and serviceability. Sample preparation as it relates to the HPLC system is discussed as are the various sample injection systems and their maintenance. A chapter discusses column selection, care, preservation, storage, and regeneration. The author points out that the detector can serve as a good trouble-shooting device (i.e., gas bubbles indicate pump check valve problems). For all the HPLC components, keeping a maintenance and trouble-shooting log book is stressed.

The author did not, however, emphasize the importance of injecting a reference standard daily to ensure proper performance of the HPLC system. This information is invaluable for daily quality control and observation of subtle system changes over long periods of time.

Overall, the author has provided a useful book to the laboratory chemist on maintaining and trouble-shooting HPLC systems.

RICHARD T. KRAUSE

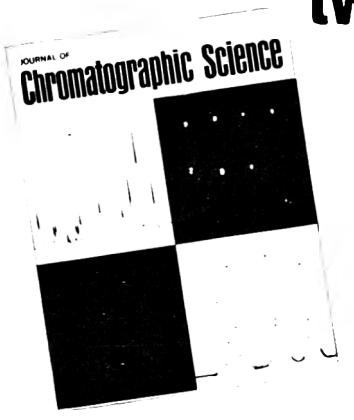
Food and Drug Administration
Division of Chemical Technology
Washington, DC 20204

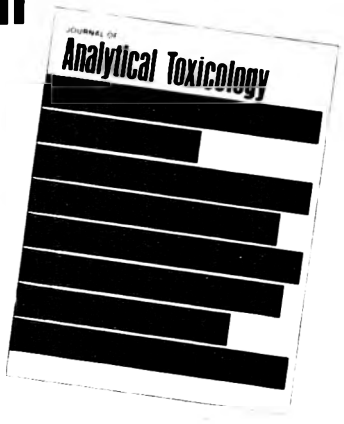
AUTHOR INDEX

SUBJECT INDEX

In This Issue

Preston Publications offers two technical journals for the analytical chemist...





Journal of CHROMATOGRAPHIC SCIENCE

The Journal of Chromatographic Science is an international scientific publication published monthly for the purpose of presenting the latest technical information concerning the practice of chromatography in all its varied forms. Every month JCS features articles describing recent advances in chromatographic techniques and applications. In addition to its featured editorial content, JCS regularly publishes announcements of new products and new literature, meetings and short courses, programs, book reviews and "news" items.

A new feature, "A Forum for Chromatographers" serves as an open invitation for analysts in the field to discuss problems they might be having in their labs. Readers' questions are forwarded to experts in the field for answers and discussions. Special issues are planned each year in order to update and provide a reference source for information in specific areas of the chromatographic field.

Journal of ANALYTICAL TOXICOLOGY

The Journal of Analytical Toxicology, a bimonthly, publishes original manuscripts, review articles and short communications relating to the isolation, identification and quantitation of potentially toxic substances and their biotransformation products in specimens of human, animal or environmental origin. Articles on the monitoring of therapeutic drugs, environmental and industrial contaminants, clinical reports of poisonings (with analytical data), the development of analytical techniques, and the results of forensic toxicologic investigations are included. JAT has new products, new literature, book reviews and meetings and short courses listings to keep the reader current.

To order your subscription, please fill out the coupon below:

I want to subscribe to:

☐ Journal of Chromatographic Science (1 yr. $62.00)

☐ Journal of Analytical Toxicology (1 yr. $67.00)

☐ Both Journals (1 yr. $127.00)

☐ Check enclosed. ☐ Please bill me.

☐ Please bill my credit card: ☐ VISA ☐ MASTERCARD

Card # _____

Expiration date _____ _____ (Signature)

Name _____

Address _____

City_____ State_____ Zip_____

Preston Publications Inc. • P.O. Box 48312 • Niles, IL 60648