ISSN 0004-5756

A O A C

MAY 1982
VOL. 65, NO. 3

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS JOURNAL

JANCA 65(3) 523–780 (1982)

How To Uncover The Hidden Costs Of Chromatography.

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

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

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

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

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

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

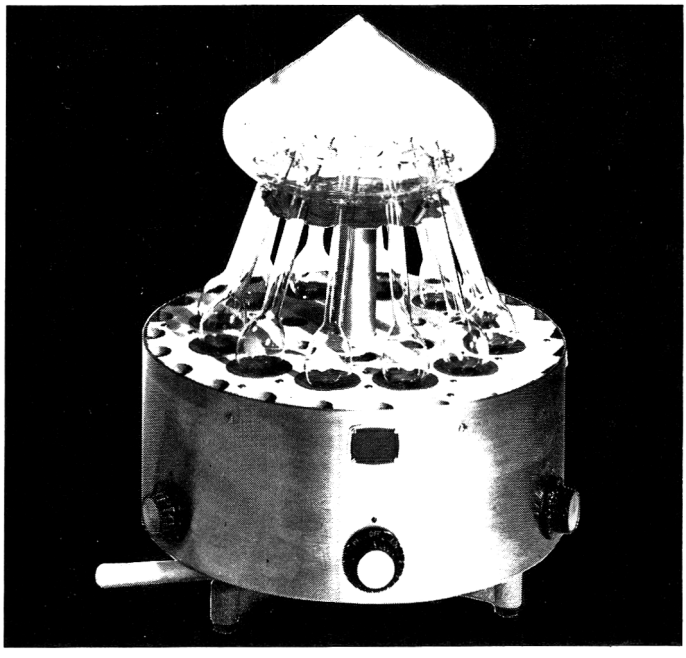
Write or call today for a free technical bulletin about B&J Brand High Purity HPLC Solvents. We'll also send you our distributor listing so you can conveniently order B&J Brand Solvents from a distributor near you.

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

BURDICK & JACKSON LABORATORIES, INC.

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

A Subsidiary of Hoffmann-LaRoche Inc.



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

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

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

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

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

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

*Application papers include use with plant and biological specimens.

KONTES

SCIENTIFIC GLASSWARE / INSTRUMENTS

Vineland, NJ 08360 (609) 692-8500

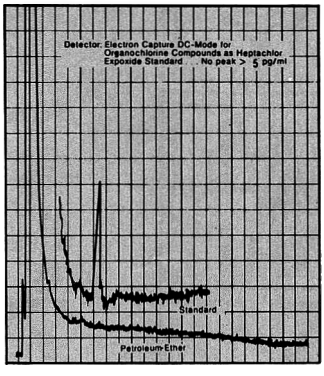
Regional Distributors: KONTES OF ILLINOIS, Evanston, Ill. • KONTES OF CALIFORNIA, San Leandro, Calif.

SPECIALLY PURIFIED SOLVENTS FOR ORGANIC RESIDUE ANALYSIS

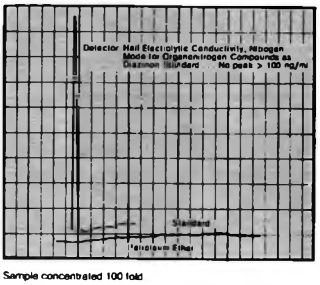
Every 'BAKER RESI-ANALYZED'® Solvent is glass distilled and use-tested to meet the exacting requirements of organic residue analysis by gas chromatography (GC).

All 17 solvents are fully characterized for key modes of GC detection. Use tests include assessment of organic residues by ECD, FID, FPD for P and S and Hall detector for N. The solvents meet the requirements of the EPA and AOAC for pesticide-residue analysis.

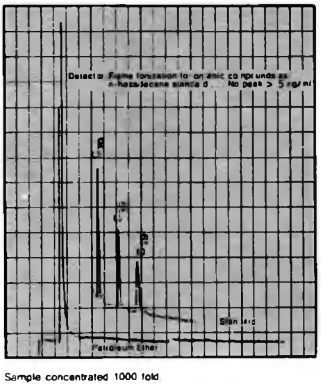
Specify 'BAKER RESI-ANALYZED'® -your assurance of interference-free solvents for gas chromatography. See J.T. Baker's new Catalog 80 for complete details. Call Baker at (201) 859-2151 to order your copy today.



Sample concentrated 100 fold



Sample concentrated 100 fold



Sample concentrated 1000 fold

8 pt(3.8 L) **3-9265**

Petroleum Ether
'Baker Resi-Analyzed'®

30-60°C
For Organic Residue Analysis

'BAKER ANALYZED' *Reagent*

LOT **024085**

Organic Residue Analyses		
Organochlorine Compounds (as heptachlor epoxide)(by GC, ECD)	no peak >	5 pg/ml
Organophosphorus Compounds (as parathion)(by GC, FPD phosphorus mode)	no peak >	100 pg/ml
Organosulfur Compounds (as parathion)(by GC, FPD, sulfur mode)	no peak >	3 ng/ml
Organonitrogen Compounds (as diazinon)(by GC, Hall detector nitrogen mode)	no peak >	100 ng/ml
FID Active Compounds (as n-hexadecane)(by capillary GC)	no peak >	5 ng/ml
Acidity	passes test	
Color (APHA)	10	max
Non-Volatile Matter	5 ppm	max
Water (H_2O)(by Karl Fischer titrn)	0.05	%max

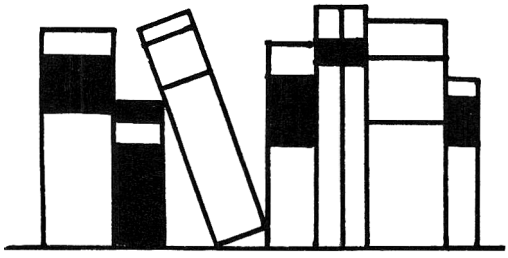
This solvent has been specially purified by a multi-step process that includes distillation in all-glass equipment.

J.T. Baker Chemical Co., Phillipsburg, N.J. 08865

J.T. Baker Chemical Co.
222 Red School Lane
Phillipsburg, NJ 08865

J.T.Baker® CHEMICALS

IS YOUR AOAC LIBRARY COMPLETE?



Official Methods of Analysis, 13th Edition. 1980. 1038 pp. Hardbound. $75 + $3 book post and handling in U.S., $75 + $6 outside U.S. ISBN 0-935584-14-5.
Compendium of over 1500 validated methods of analysis for foods, drugs, cosmetics, plants, feeds, fertilizers, hazardous substances, water, and other products affecting agriculture and public health and safety.

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.

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 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 MAY 1982 No. 3

CONTENTS

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

INFORMATION FOR SUBSCRIBERS AND CONTRIBUTORS

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

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

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

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

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

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

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

INSTRUCTIONS TO AUTHORS

Scope of Articles

The Journal of the AOAC will publish articles that present, within the fields of interest of the Association (*a*) unpublished original research; (*b*) new methods; (*c*) further studies of previously published methods; (*d*) background work leading to development of methods; (*e*) compilations of authentic data; (*f*) technical communications, cautionary notes, and comments on techniques, apparatus, and reagents; (*g*) invited reviews of methodology in special fields. All articles are reviewed for scientific content and appropriateness to the journal.

Preparation of Manuscript

Authors are required to submit three copies of the complete manuscript, including all tables and all illustrations. The manuscript is to be typewritten on one side only of white bond paper, $8\frac{1}{2} \times 11$ inches, with minimum page margins of 1 inch, and must be **double-spaced** throughout (including title, authors' names and addresses, footnotes, tables, references, and captions for illustrations, as well as the text itself). Tables are to be typed on separate sheets, *not* interspersed through the manuscript. Drawings and photographs should be mounted apart from the text or submitted as separate items, *not* interspersed through the text.

Style and Format

The text should be written in clear, concise, grammatical English. Unusual abbreviations should be employed as little as possible and must always be defined the first time they appear. Titles of articles should be specific and descriptive. Full first names, middle initial (if any), and last names of authors should be given. The address of the institution (including zip code) from which the paper is submitted should be given and should be in a form to which inquiries, proofs, and requests for reprints can be sent. Information supplementing the title and authors' names and addresses should be given in footnote form.

Methods, Results and/or Discussion, Acknowledgments, and Recommendations (applicable to reports of General and Associate Referees) should be placed in separate sections under appropriate headings typed in capitals and lower case letters, centered on the page, *not* underscored.

Abstracts: Each manuscript should be accompanied by a concise abstract (not more than 200 words). The abstract should provide specific information rather than generalized statements.

Introduction: Each article should include a statement on why the work was done, the previous work done, and the use of the compound being studied.

Methods: Methods should be written in imperative style, i.e., "Add 10 mL . . . Heat to boiling . . . Read in spectrophotometer." Special reagents and apparatus should be separated from the details of the procedure and placed in sections with appropriate headings; however, common reagents and apparatus (e.g., concentrated HCl, chloroform, ordinary glassware, ovens, etc.), or those which require no special preparation or assembly, need not be listed separately. Hazardous and/or carcinogenic chemicals should be noted. The steps of the procedure should not be numbered, but should be grouped together to form a logical sequence of two, three, or four operations. Any very long, detailed operation can be given in a separate section with an appropriate heading (e.g., Preparation of Sample; Extraction and Cleanup; Preparation of Standard Curve). Any necessary calculations should be included. Care should be taken that the number of significant figures truly reflects the accuracy of the method. Equations should be typed in one-line form. Wherever possible, metric units should be used for measurements or quantities.

Tables: All tables must be cited in the text consecutively. Tables are numbered by arabic numbers, and every table must have a descriptive title, sufficient so that the table can stand by itself without reference to the text. This title should be typed in lower case letters, *not* capitals, with the exception of the word "Table" and the first word of the descriptive portion of the title, of which the first letter is capitalized. Every vertical column in the table should have a heading; abbreviations may be used freely in the headings to save space, but should be self-evident or must be explained in footnotes. Footnotes to both the headings and the body of the table are indicated by lower case letters in alphabetical order; these letters should be underscored and raised above the line of type. Horizontal rules should be used sparingly; however, they are used to bound the table at top and bottom and to divide the heads from the columns. Authors should refer to recent issues of the Journal for acceptable format of tables; tables should not exceed the normal page width of the Journal, and authors should attempt to revise or rearrange data to fit this pattern.

Illustrations: Illustrations, or figures, may be submitted as original drawings or photographs; photocopies are acceptable for the two review copies but not for the printer's copy. All figures must be cited in the text consecutively. Figures are numbered by arabic numbers, and all figures must be accompanied by descriptive captions, typed on one (or more) separate sheets, *not* on the figure itself. The figure should be identified by number on the back by a soft pencil or (preferably) a gummed label.

Drawings should be submitted either as the original drawing or a good glossy photograph; photocopies, multiliths, Verifax copies, Xerox copies, etc. are *not* acceptable. Drawings should be done in black India ink (ordinary blue or blue-black ink is not acceptable) or with drafting tape on white tracing paper or tracing cloth or on "fade-out" graph paper (ordinary graph paper ruled with green or dark blue ink is not acceptable). Lettering should be done with a Leroy lettering set, press-on lettering, or a similar device; freehand or

typewritten lettering is not acceptable. Values for ordinate and abscissa should be given, with proper identification conforming to journal style (example: wavelength, nm), at the sides and bottom of the figure. Lettering or numbering on the face of the figure itself should be kept at a minimum; supplementary information should be given in the caption. Several curves on the same figure should be identified by simple symbols, such as letters or numbers, and the proper identification or explanation given in the caption. Letters and numbers should be large enough to allow reduction to journal page or column size. *JAOAC* does not publish straight line calibration curves; this information can be stated in the text. The same data should not be presented in both tables and figures.

Footnotes: Footnotes are a distraction to the reader and should be kept to a minimum. Footnotes to the text are identified by arabic numbers set above the line of type (not asterisks or similar symbols). Each footnote must be indicated by its number within the text.

Acknowledgments: Essential credits may be included at the end of the text but should be kept to a minimum, omitting social and academic titles. Information on meeting presentation, financial assistance, and disclaimers should be unnumbered footnotes and appear after the *References* section.

References: References to previously published work should be collected at the end of the article under the heading "References." Each item in the list is preceded by an arabic number in parentheses. Every reference must be cited somewhere in the text in numerical order (rather than alphabetical or chronological). (*Note:* If an article contains only one reference, this reference may be inserted directly in the text, rather than placed at the end.) It is the author's responsibility to verify all information given in the references.

References to journal articles must include the following information: last names and at least one initial of *all* authors (*not* just the senior author); year of publication, enclosed in parentheses; title of journal, abbreviated according to accepted *Chemical Abstracts* style; volume number; numbers of first and last pages. References to books, bulletins, pamphlets, etc. must include the following information: last names and initials of authors or editors; year of publication, enclosed in parentheses; full title of book; volume number or edition (unless it is the first edition); publisher; city of publication; numbers of pertinent pages, chapter, or section. Citation to private communications or unpublished data should be included in the text, *not* in the list of references, in the following form: author's name and affiliation, and year.

The abbreviation for the journal title should be repeated for each reference; do not use *ibid.* *This Journal* will be referred to as *J. Assoc. Off. Anal. Chem.*

The compendium of methods of the Association should be listed as follows: *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, with appropriate section numbers; the edition and year are, of course, subject to change.

Symbols and Abbreviations

kg	kilogram(s)
g	gram(s)
mg	milligram(s)
μg	microgram(s)
ng	nanogram(s)
L	liter(s)
mL	milliliter(s)
μL	microliter(s)
m	meter(s)
cm	centimeter(s)
mm	millimeter(s)
μm	micrometer(s) (*not* micron)
nm	nanometer(s) (*not* millimicron)
A	ampere(s)
V	volt(s)
dc	direct current
ft	foot (feet)
in.	inch(es)
cu. in.	cubic inch(es)
gal.	gallon(s)
lb	pound(s)
oz	ounce(s)
ppm	parts per million
ppb	parts per billion
psi	pounds per square inch
sp gr	specific gravity
bp	boiling point
mp	melting point
id	inside diameter
od	outside diameter
h	hour(s)
min	minute(s)
s	second(s)
%	percent
$\bar{s}$	standard taper
N	normal
M	molar
mM	millimolar

(Note: Spectrophotometric nomenclature should follow the rules contained in *Official Methods of Analysis*, "Definitions of Terms and Explanatory Notes.")

How To Comply With The New Copyright Law

Participation in the Copyright Clearance Center (CCC) assures you of legal photocopying at the moment of need.

Libraries everywhere have found the easy way to fill photocopy requests legally and instantly, without the need to seek permissions, from more than 3000 key publications in business, science, humanities, and social science. You can:

Fill requests for multiple copies, interlibrary loan (beyond the CONTU guidelines), and reserve desk without fear of copyright infringement.

Supply copies from CCC-registered publications simply and easily.

The Copyright Clearance Center is your one-stop place for on-the-spot clearance to photocopy for internal use.

Its flexible reporting system accepts photocopying reports and returns an itemized invoice. You send only one convenient payment. CCC distributes it to the many publishers whose works you need.

And, you need not keep any records, the CCC computer will do it for you. Register now with the CCC and you will never again have to decline a photocopy request or wonder about compliance with the law for any publication participating in the CCC.

To register or for more information, just contact:

Copyright Clearance Center
21 Congress Street
Salem, Massachusetts 01970
(617) 744-3350

a not-for-profit corporation

NAME _____ TITLE _____

ORGANIZATION _____

ADDRESS _____

CITY _____ STATE _____ ZIP _____

COUNTRY _____ TELEPHONE _____

AOAC METHODS DEVELOPMENT—CHALLENGE OF THE NEXT DECADE

95th Annual Meeting of the AOAC, October 1981

This symposium is being held in commemoration of the 75th Anniversary of the enactment of the Pure Food and Drugs Act and the Wholesome Meat Inspection Act. We will look briefly at the situation in 1906 with respect to regulatory analysis. Then Dr. Horwitz will review the development of the collaborative study. Three subject area specialists, Drs. Tanner, Zarembo, and Feldman, will discuss the challenges of the 1980s in their areas of chemical food analyses, chemical drug analyses, and microbiological analyses. Mr. Levine, an attorney, will speak on the relationship of sound methodology and regulatory action.

In looking back a century, one finds reports of confusion and frustration with respect to "official" analyses of foods and drugs. In 1875, the British Parliament passed the Sale of Food and Drugs Act which made the appointment of analysts mandatory. Reportedly, "Endless disputes unavoidably arose, friction with manufacturers and traders .... Conflicting decisions came to the various benches of magistrates upon similar cases ... rendered the position of the merchants unsatisfactory. It was not recognized by Parliament until almost a quarter of a century had elapsed that it was not enough to compel local authorities to get samples analyzed, but that it was also the duty of the Parliament to lay down specific and clear instructions that might enable the officers to do their work." Thus, legislation providing for the performance of testing must also provide for standardization of the testing methods.

At the turn of the century, Dr. Harvey W. Wiley sensed the need for a means of standardizing tests and specifications to be used in regulation. His answer was the Association of Official Agricultural Chemists and the referee-collaborative study concept whereby a proposed method would be tested in every detail in several independent laboratories reporting concordant results. Such a collaborative study would constitute the ultimate means for establishing the scientific validity of an analytical method. In his autobiography, Dr. Wiley wrote of the AOAC, "Up to the formation of this association the methods used in the chemistry of agriculture were crude, inconclusive and in many cases erroneous. An agricultural product analyzed by two or more chemists would present very frequently many important variations. If products were sold on an analysis of this kind the purchaser might be defrauded in one case and the seller might be defrauded in another. In either case chaos reigned. The work of the AOAC has brought order out of this chaos." Certainly, current standards for the uniformity of foods, drugs, and fertilizers would be impossible without the interlaboratory, collaborative mechanism.

In reviewing the five presentations of this symposium, it becomes readily apparent that there is indeed a challenge of the 1980s. Modern, sophisticated instrumentation, including automatic data processing, will be required in all three areas discussed. In both foods and drugs, there will be need to detect and identify materials at concentrations not previously possible. Much of the equipment to be used requires specialized operator training and thus the old concept of all chemists in a laboratory being able to perform any official method is passé. Yet, the concept of standardization through interlaboratory collaborative study will continue to occupy a position of great importance. The different parties involved in regulation must each be able to arrive at equivalent conclusions when analyzing a given sample. As is pointed out in the symposium, where highly specialized equipment is involved, getting a full complement of collaborators may be difficult. Perhaps

there should be more flexibility in the criteria used for establishing the "official" statuses.

The researcher at the bench will discern from these papers that he should keep up the important work of developing methods. There should be no hesitation in using elaborate equipment when it is needed, but a quotation from the noted analytical chemist I. M. Kolthoff should be heeded: ". . . there are potential drawbacks in an approach to analytical problems in which too much emphasis is placed on apparatus and data processing and not enough on the chemical principles behind the measurement being made." If measurements are not meaningful, then the work is in vain. A specific example of the truth of this admonishment can be found in an occurrence with high molecular weight antibiotics. For years, commercial amphotericin was assumed to be composed of "A" and "B" fractions, the "B" being the one of therapeutic value. Commercial samples were tested for "A" content and a limit imposed. With the advent of greater chromatographic resolution, it was found that the "B" fraction consists of 2 principal components (M. Margosis and A. Aszalos, Food and Drug Administration, 1981). Thus, the whole situation must now be re-examined, some experimental work done, and new specifications established.

It is AOAC's opportunity and obligation to continue its role of leadership in seeing that the necessary methods are developed, properly scrutinized, adopted, and publicized

THOMAS G. ALEXANDER

Food and Drug Administration
National Center for Antibiotics Analysis
Washington, DC 20204

Evaluation of Analytical Methods Used for Regulation

WILLIAM HORWITZ
Food and Drug Administration, Bureau of Foods, Washington, DC 20204

Regulatory analysis requires methods for analysis for 3 purposes: surveillance, to detect problems; monitoring, to follow trends; and compliance, to enforce specifications (standards, action levels, tolerances). Each purpose requires a different weight to be assigned to the relative importance of the scientific characteristics of methods of analysis: systematic error, repeatability, reproducibility, limit of determination, and specificity. Evaluation consists of balancing the level of scientific requirements against the practical considerations of cost, time, and level of training required. In trace analysis, FDA utilizes recovery limits of 80-110% at ≥0.1 ppm and 60-100% below. The criterion for acceptable reproducibility (total of between- and within-laboratories) of methods of analysis can be represented by a relationship between coefficient of variation and concentration. The results of collaborative studies show that 10% outliers must be tolerated, but above 20% is excessive. Other new, important criteria for evaluation of methods at trace levels are % false positives, false negatives, and the magnitude of the blank.

For almost 100 years the Association of Official Analytical Chemists (AOAC) has been conducting collaborative studies to obtain information on the reliability of analytical methods used to support regulatory actions. The information sought is the allowance to be permitted for differences in assay results between laboratories in determining compliance with a legal requirement. The design of such studies is deceptively simple, as overseas organizations, in the process of instituting similar programs, are discovering. Considerable preparation is required to be sure that a method is ready for a collaborative study. The test parameters must be carefully chosen to cover the range of samples and concentrations of interest and to provide data amenable to statistical analysis for the required method attributes. Then the results of the study must be suitably interpreted. Despite the long history of practical use of collaborative studies as the basis for approval of methods of analysis for regulated commodities, the AOAC has never prepared a treatise to formalize procedures for the design, conduct, and interpretation of interlaboratory studies. Recognizing this deficiency, the President of the AOAC has now appointed a Committee on Collaborative Studies to produce such a manual.

Although we know intuitively what is desired, it is difficult to express this knowledge in an unambiguous way. Our first requirement is a statement of the desired end product. The usual answer is, "The best method possible—one that is accurate, precise, specific, and rapid." As practical chemists we know it is impossible to maximize all of these characteristics simultaneously. Nor do we need to. In regulatory work there are usually three major purposes for which we need analytical values: (*1*) to survey a field to determine the extent of a problem; (*2*) to monitor trends to determine if corrective action is required; and (*3*) to determine compliance with a legal or economic specification. Each of these purposes requires that a different emphasis be placed on the various characteristics of methods. In surveying a field, the normal variability of a commodity and the vagaries of bulk sampling are so large that a high degree of accuracy and precision in the method is usually unnecessary; speed is most likely the desired attribute. In monitoring trends, or changes in a value with time, systematic error, as long as it is constant, is unimportant, but the precision must be good enough to detect the changes to be measured. In compliance activities, accuracy (lack of bias) and precision are important, but only at the specification level, unless the specification is based on the method itself, in which case accuracy is unnecessary by definition. Each of these purposes requires a different mix of characteristics, what is best for one purpose may not be best for another. Therefore, there is no such thing as a "best method." Methods must be evaluated with their purpose in mind. Usually the purpose is not known beforehand, so we assume that a high degree of reliability (good reproducibility and low bias) is needed, since it is almost always easier to simplify a method by eliminating steps not necessary for a desired purpose, than it is to insert steps to eliminate sources of errors. However, regardless of the application, the method must be capable of producing measurements that are in statistical control. By statistical control is meant (1) that the

This paper was presented at the Symposium on AOAC Methods Development—Challenge of the Next Decade, 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

0004-5756/82/6503-0525-06$01.00

variability of data from a measurement process is sufficiently stable that the process can be relied on to produce results with a predicted variability at a stated level of confidence. It does not matter whether the error is large or small as long as it consistently lies within the interval predicted by the statistical parameters.

To determine if a measurement process, such as an analytical method, produces results which are in statistical control, it is necessary to make a sufficient number of measurements under the conditions likely to be encountered in actual practice. The method must be applied by a number of laboratories, representative of those that will eventually conduct the routine analyses, to a number of different types of samples which contain the range of concentrations to be expected. In other words, a collaborative study is needed. The collaborative study is an investment of effort to provide for each method of analysis a predicted range within which we expect future analytical values will lie. The more extensive the study, the better our predictions, but at the expense of increased cost. The problem of how much of an investment in time, samples, and collaborators can be afforded must always be faced. The products of a collaborative study should be an estimate of interlaboratory precision (reproducibility), which we define to include intralaboratory precision (repeatability) and an estimate of systematic error (bias). In special cases, information on specificity (identification) and limit of reliable measurement is also needed. In almost all cases, the interlaboratory precision is the most important product, since frequently it also determines the boundaries of the other properties.

Interlaboratory Precision

From the point of view of determining compliance with a regulatory specification or tolerance, the most important characteristic of a method is the interlaboratory precision. This is a measure of the variability in results from different laboratories that examine presumably identical test materials. In general, the answer is a function of two primary factors: How good is the data base and how sure do we want to be of being right; or stated conversely, how much risk do we wish to take of being wrong? If there is no control over the number and quality of laboratories that will be producing values, the data base should include as large a representation of laboratories as practicality will permit. If only a few laboratories will be called upon to verify the findings, these few laboratories can constitute the entire population supplying information, eliminating any error in the sampling of laboratories for a collaborative study. The second factor, the risk of being wrong, has to some extent been standardized by the statisticians. They ordinarily accept a 5% risk of being wrong as reasonable. The next standardized level of risk is usually 1%. If only a few samples, say 3 or 5, are run from a given batch or consignment, the likelihood of being wrong (or being outside of limits) is acceptably small when the risk is 1 in 20 or greater; but if 20 samples are customarily run, or if 20 laboratories customarily participate in a proficiency trial, one of them will routinely be expected to be outside of limits in each trial. This may not be acceptable. In the latter case, a 1% risk level is more likely to be chosen, but at the expense of a greater allowance for the expected variability between laboratories.

In determining what constitutes acceptable precision, we can use the curve derived from previous collaborative studies that relates between-laboratory precision, expressed as coefficient of variation, to concentration (2). This curve, which is now a smoothed summary of over 200 independent collaborative studies covering numerous AOAC topics from pharmaceutical preparations and pesticide formulations at the high end to trace elements and aflatoxins at the low end of the concentration scale, is represented by the following equation:

$$CV = 2^{(1-0.5 \log C)}$$

where CV is the between-laboratory coefficient of variation expressed in percent and concentration is expressed in negative powers of 10. An easily remembered reference point, applicable to pesticide residues and trace elements, is that an interlaboratory CV of 16% (2^4) can be expected at a concentration of 1 ppm (10^{-6}). It is interesting that this curve appears to be independent of the nature of the analyte, the matrix, and the measurement technique. It is possible that this curve merges with physical measurements on the high concentration end and with biological measurements on the low concentration end, if there were some way all of these measurements could be expressed in the same units.

Since this curve is only an estimate of a statistical parameter, it is bounded by an upper and lower confidence limit, calculated from the following standard error function of the CV, expressed as a fraction (3):

$$SE_{CV} = CV \sqrt{(1 + 2\,CV^2)/2n}$$

The 95% confidence interval is approximately

Table 1. 95% Confidence limits of the coefficient of variation (CV) taken from the general CV/concentration curve at important concentration levels

Concn	Analyte	CV, %	95% Confidence limits of CV	
			1 Study	10 Studies
100 ppm	Drugs in feeds	8	0–19	6–10
1 ppm	Pesticide residues Trace elements	16	0–39	12–20
10 ppb	Aflatoxins Nitrosamines	32	0–82	23–41
100 ppt	Dioxin Aflatoxin M	64	0–186	42–86

twice the standard error. Table 1 gives some examples of the calculated confidence interval for some important points on the general CV/concentration curve for $n = 1$ and $n = 10$ studies. From the table it is obvious that a single study provides a very poor estimate of the reproducibility of a method of analysis.

Because of the expense of collaborative studies, we may have to accept the fact that if the reproducibility of a method in a collaborative study approximates the general CV/concentration curve, the method is acceptable. Numerous illustrations of the adherence of method parameters to the general CV/concentration curve are available and a number of patterns of interlaboratory data superimposed on the general curve are given in a recent paper (4). These examples cover the macro concentration scale, from fat in meat by gravimetric analysis and methyl esters of fatty acids by gas chromatography to the trace concentration levels of pesticide residues by gas chromatography, trace elements by atomic absorption spectrophotometry, and aflatoxins by thin layer chromatography. Considerable data exist on the performance of aflatoxin methods by thin layer chromatography at the low parts per billion concentration levels (5).

These data suggest that one criterion for the acceptability of methods is conformity to the general CV/concentration curve, with due consideration to its confidence limits. There may be occasions in the absence of better methods where poorer performance than that derived from the general curve must be accepted. This poorer performance, or performance at the higher extreme of the confidence interval, should indicate that the method could be improved, at least to the point of matching the general curve fairly well. Better performance than that derived from the general curve would suggest that attempts to improve the precision of the method might not be profitable, although there may be considerable room for improvement with respect to other characteristics such as recovery, speed, and economy.

It must be emphasized that *intra*-laboratory precision must not be compared with the *inter*-laboratory precision of the general curve. Intralaboratory precision is usually one-half to two-thirds that of interlaboratory precision (2).

Outliers

A characteristic which is not ordinarily observed during the course of development or routine use of a method is the propensity to produce outliers. Outliers are values which are far removed from the main body of data. The more isolated the point in terms of distance from the main body of data, and the greater the number of points in the main body of data, the greater the probability the isolated point is a true outlier. Outliers are rarely reported in published papers from a single laboratory since they are either ignored or removed by repetition. In interlaboratory studies, however, values cannot be identified as being either consistent with the main body of data or as outliers until a considerable amount of data has been accumulated. At this point, when outliers are present they seem to form a consistent, irreducible pattern of 5–15% of the reported values, usually isolated individual values but occasionally all of the values on a series of samples from a laboratory. "Irreducible outliers" here means outliers for which a cause cannot be ascertained, since some outliers, when called to the attention of the reporting laboratory, are found to be caused by calculation errors or incorrect standards, and are thus remediable.

The outlier rate seems to be more or less independent of the acceptable working concentration ranges, with perhaps a slight increase at the lower levels. Then as the limit of determination approaches, outliers at the low side begin to accumulate at zero as false negatives, a new category of method evaluators.

In AOAC collaborative studies the maximum number of outliers which must be tolerated is established by the Youden rule of requiring a minimum of 5 laboratories per study. If 1 of 6 laboratories appears as a consistent rank outlier and if no additional outliers appear in the data from the other laboratories, the outlier rate is automatically limited to 17%. It follows that a study with a bare minimum of 5 laboratories is risking the possibility that the method will be rejected because of too many outliers among too few laboratories. A minimum of 7 laboratories would permit a buffer of 1 rank outlier and a scattering of individual outliers without risking the rejection of a method because of too few laboratories for statistical analysis of the data. In trace analysis, the production of a consistent pattern of outliers by a single laboratory is not at all ususual, particularly in the early stages of acquiring experience with a new technique, or where standards are of variable quality and stability. Naturally, if a consistent pattern of outliers appears from a single laboratory, a diligent search for the cause should be initiated, so that this uncontrolled source of error can be noted and, where possible, precautions instituted.

At present, AOAC statisticians are applying the Youden ranking test (6) to laboratories and the Dixon range test to individual values and laboratory averages, but no systematic investigation has been performed to determine the relative value of these and the several dozen other statistical tests for outliers that are available (7).

False Positives and False Negatives

As a method is studied close to its limit of determination, the outliers at the low side begin to accumulate at zero. When a known amount of analyte has been added, these zeros are definitely false negative values (the analyte is known to be present but is reported to be absent). This shift in the distribution of interlaboratory results as the limit of determination is approached is evident in the aflatoxin methods proficiency study data of Friesen et al. (8). If the figures showing the distribution of reported values are arranged in order from high to low mean concentrations, it is seen that the frequency distribution of reported values shifts from approximately a normal distribution at the highest values through a bimodal distribution with one peak at zero and another at a positive value, to a single peak at zero falling off sharply to positive values. There are no negative values (the blank greater than the determination) because aflatoxin analyses require no blanks. (The clear plate is automatically set at zero.) In the case of the bimodal distribution, it is not possible to assign a mean value to the analytical determination because some laboratories are reporting a definite value and others are assigning a zero value. From the viewpoint of each laboratory, there is no problem; each laboratory has given an unequivocal result. Only from the vantage of the entire study is it seen that the result consists of two answers: zero and a positive value. When the percent of zero values from aflatoxin studies is plotted as a function of concentration, the curve takes on the appearance of the general CV/concentration curve. This potential evaluation function is being reviewed in greater detail with aflatoxin and other analyte studies.

Similarly, when blank samples are analyzed, positive values are sometimes reported; some of these are obviously outliers. They are also false positives (the analyte is known to be absent but is reported as a positive determined value). Here, too, the results from each laboratory's viewpoint are unequivocal until they are examined as part of a total interlaboratory picture.

False positive values can appear in the blank determinations from any method operating at any concentration level. False negative values are rare at the higher concentration levels but increase as the method approaches the limit of determination and goes out of statistical control. If the concentration levels are appropriately spaced in an interlaboratory study, the limit of determination of a method can be determined merely by inspection of the trend of false negative values. This was demonstrated (9) in the results of an EPA interlaboratory study of the recovery of dioxin from standards, beef fat, and human milk. Most of the false negative reports occurred at 9 ppt and lower levels. No false negatives were reported in the beef fat and human milk samples above 9 ppt. There was a very sharp distinction in the characteristics of the values as a group above and below the 9 ppt point. This same value of 9 ppt was arrived at in an independent paper published by one of the participants in the study which discussed only his own values, making "use of rigorous statistical design and analysis of the data" (10).

Therefore an evaluation of the false positive and false negative patterns of the data may provide a new characteristic of the performance of methods delineating the limit of measurement.

Systematic Error

Systematic error or bias is usually determined through recovery measurements of known amounts of analyte added to a presumably negative matrix. This procedure is not entirely satisfactory since some analytes do not behave the same when added artificially as compared to natural deposition. Nevertheless, the Food and Drug Administration has proposed (11) that average within-laboratory recoveries of 60–110% will be acceptable at concentrations of less than 0.1 ppm and 80–110% at or above 0.1 ppm. Naturally, better recoveries would be preferred, but these figures seem to represent practical values in trace analysis. Lower recoveries can be accepted in special cases, provided they are reproducible or the method utilizes internal standards, particularly isotope dilution procedures. Recoveries that differ between laboratories are obviously a part of the interlaboratory random error; when this variability is too large it is impossible to determine the true systematic error of a method. Currie and DeVoe (12) point out that 15 observations are required merely to detect a systematic error of the same magnitude as the standard deviation. Therefore, to detect recoveries that are systematically and significantly less than 85% in the pesticide residue region (about 1 ppm), the experiment must contain at least 15 independent observations. Obviously the random error of precision limits the determination of systematic error. In general, if the interlaboratory precision is acceptable, the systematic error will also be acceptable.

Other Attributes

The specificity and limit of reliable measurement are other attributes of analytical methods which must be emphasized under special circumstances. Specificity is the property of responding exclusively to the material to be measured. It is especially important when structurally similar chemicals may be present which have considerably different biological properties. It is achieved in many methods of analysis by a chemical or physical "cleanup" step followed by a measurement step which may or may not be sufficiently specific. Many similar compounds may be present at trace levels which may respond like the material sought. In addition, so many homologs and isomers must be distinguished from the desired compound, particularly in the low ppb and below level, that it is frequently necessary to apply additional identification steps, designated as "confirmation of identity." A number of collaborative studies of methods for aflatoxins have been performed with specificity as the particular objective. The ultimate decision to accept a confirmation of identity method is made on the basis of the same statistical parameters used for finding the limit of determination, i.e., observations for false positives (reporting the material as present when it is not) and false negatives (not reporting the material when it is present).

With extremely toxic materials such as dioxin and aflatoxin, it is necessary to have methods to cover all biologically significant concentrations. Despite the considerable literature on the limits of measurement (13), there is little understanding or agreement on how to determine the limit. The influence of the interlaboratory environment on measurements at the limit has been entirely ignored in this literature. The limit of reliable measurement has been reached when a method goes out of statistical control within a laboratory and even sooner (in terms of higher concentration) when there is disagreement among laboratories as to the presence or absence of the compound sought at any concentration, even though each laboratory is convinced as to the correctness of its observations. A method can be accepted for use only over the range where there is substantial agreement among laboratories. Therefore, interlaboratory precision will determine the limit of measurement.

In the special case where "none" of the analyte is permitted, the regulatory limit subsequently set is often directly related to the level of acceptable performance of the method.

There also may be cases, particularly in the early stages of an investigation, where there is a real need to conduct analyses and make decisions which cannot be postponed until a method is available which meets all of the acceptability criteria. Then the "best" method available must be used, keeping in mind its limitations.

Summary

The following criteria may be useful for evaluating the acceptability of methods of analysis for compliance with a legal specification or tolerance, which places the tightest requirements on minimizing bias and and random error: (*1*) an interlaboratory coefficient of variation in percent which approximates, or is better than, that given by the equation: $CV(\%) = 2 \exp(1 - 0.5 \log C)$, where the concentration, C, is expressed in negative powers of 10; (*2*) recoveries 80–110% at or above 0.1 ppm and 60–110% at less than 0.1 ppm; (*3*) an outlier rate within 5–15% but

no greater than 20%, when at least 5 laboratories participate in the collaborative study; and (4) no more than an acceptable number, yet to be determined, of false positives and false negatives. In cases where potential interference or closely related less toxic compounds may be present, a confirmation of specificity at the specification level may also be necessary.

References

(1) Wernimont, G. (1977) in *Validation of the Measurement Process*, James R. DeVoe (Ed.), ACS Symposium Series 63, American Chemical Society, Washington, DC, pp. 1–29

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

(3) Weatherburn, C. E. (1969) *A First Course in Mathematical Statistics*, Cambridge University Press, London, England, p. 144

(4) Horwitz, W. (1982) *Anal. Chem.* **54**, 67A–76A

(5) Horwitz, W., & Albert, R. (1982) *Assoc. Food Drug Off. U.S. Q. Bull.* **46**, 14–24

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

(7) Barnett, V., & Lewis, T. (1978) *Outliers in Statistical Data*, John Wiley and Sons, New York, NY

(8) Friesen, M. D., Walker, E. A., & Castegnaro, M. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1057–1066

(9) Horwitz, W. (1981) in *The Pesticide Chemist and Modern Toxicology*, S. Kris Bandel et al. (Eds.), ACS Symposium Series 160, American Chemical Society, Washington, DC, pp. 411–438

(10) Gross, M. L., et al. (1981) *Anal. Chem.* **53**, 1902–1906

(11) Food and Drug Administration (20 Mar. 1979) *Fed. Regist.* **44**, 17070–17114

(12) Currie, L. A., & DeVoe, J. R. (1977) in *Validation of the Measurement Process*, James R. DeVoe (Ed.), ACS Symposium Series 63, American Chemical Society, Washington, DC, pp. 114–139

(13) Liteanu, C., & Rica, I. (1980) *Statistical Theory and Methodology of Trace Analysis*, Halstead Press, A Division of John Wiley and Sons, New York, NY

Analytical Methods for Foods in the Next Decade

JAMES T. TANNER
Food and Drug Administration, Division of Nutrition, Washington, DC 20204

With each passing decade new problems arise for the regulatory analytical chemist. The push for low detection limits from percent to parts per million to parts per billion brought the need for new and improved analytical instrumentation followed by questions of reliability at such low values. Each question has been met by new instruments or techniques and critical studies. The question for the 1980s is not how to achieve low detection limits but how to reliably and rapidly perform analyses at low values. During the 1960s the emphasis was on the single component/element techniques. We seem now to be entering the computer-controlled era. In each analytical specialty, computer-controlled instruments are offered which greatly aid the analyst in producing an accurate, reliable analysis in a shorter time. The advantage of larger numbers of analyses per unit of time with, in some cases, reduced personnel are not to be overlooked in this age of economy. To the AOAC collaborative study this means a reduction in the number of laboratories who can participate. It also means greater standardization of methodology, and the chemist's laboratory ability becomes less of a factor in producing reliable analyses. Specific analytical examples are discussed to illustrate the trend for the 1980s.

The workday of an analytical chemist in the laboratories of the 1980s is quite different from that of the traditional analytical chemist of yesterday. We can trace this change over the past several decades by looking at examples of instruments which coincide with this development.

During the 1960s and before, analytical chemistry was a combination of traditional "wet" chemistry together with a variety of instrumental methods for the final determinative step. The emphasis was on the separation of the element or compound to be determined by classical chemical techniques—solvent extraction, ion exchange, precipitation—and then the final determination of that element or compound on some suitable instrument. The results were then calculated by hand, based on the readout from the instrument. The determination was very time consuming, and frequently the accuracy and precision of the value obtained was a function of the skill of the laboratory analyst.

During the 1970s for several reasons the emphasis shifted from analyzing a few samples and drawing conclusions to analyzing a large number of samples (surveillance). The discovery of contaminants such as mercury, DDT, polychlorinated biphenyls, and lead in the environment led to large surveys in which hundreds or thousands of samples had to be assayed on a rapid time scale. The traditional methods were inadequate to keep up with the demand. New instruments, some developed for specific analyses, were introduced. The emphasis also shifted from the single component to multicomponent analyses, enabling the chemist to produce more analyses per unit time. Some instruments began to appear in which automatic processing capabilities were built in, thereby speeding up the analyses and eliminating some of the variability of results due to differing laboratory skills.

In addition, the analyst was asked to lower the limits of detection to less than part per million levels. The public's concern with minute traces of some contaminants and the necessity of analyzing large numbers of samples placed a considerable burden on the analyst of the 1970s.

We are now in the 1980s and the question is, what can we expect from the instrument producers during this decade? I believe that this will be the decade of automation. The instruments of the 1970s will be continued, but they will be controlled by mini- or microcomputers. Functions such as calibration, graphics, and data reduction will be routinely done "on-line." Consequently the throughput will be increased and analyst variability will be minimized.

To illustrate these trends, we will look at several techniques and their development over the years, and then examine what this will mean to AOAC, the collaborative study, and incorporation of verified methods into *Official Methods of Analysis* of the AOAC.

Neutron Activation Analysis

During the 1960s neutron activation analysis (NAA) was used extensively for elemental analysis. The emphasis was on a clean radiochemical separation of the element of interest

This paper was presented at the Symposium on AOAC Methods Development—Challenge of the Next Decade, 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

0004-5756/82/6503-0531-04$01.00

followed by a count of the radioactivity with an NaI(Tl) detector coupled to either a single channel analyzer or a small multichannel analyzer. Peak integration was done by hand and the results were calculated with a simple calculator. Few elements could be determined simultaneously.

By the mid 1970s, a Ge(Li) detector had replaced the NaI(Tl) detector, and was connected to a large multichannel analyzer with crude peak integration capability. The higher resolution Ge(Li) detectors made multielement analysis common. Although these analyzers had crude peak integration capabilities, most data reduction was accomplished through the use of large computers. During this time, many complex computer reduction programs were written and were in common use. The hand calculators were now a thing of the past.

In the late 1970s, multichannel analyzers with large memories were introduced. Although the larger memory was achievable elsewhere, the large capacity coupled with highly developed software was a major breakthrough. Also during this time, the efficiency and resolution of detectors had steadily increased. The combination produced an analytical method which required only that the sample be irradiated and placed in front of the detector. The analysis of the samples and computation of concentrations could be done virtually on-line, and a summary report could be typed at the conclusion of the day. Automatic sample changers had already been introduced which allowed the operation of the equipment on a 24-h-a-day basis.

The end result was that analytical personnel could reliably perform large numbers of analyses on a daily basis. This trend was duplicated in other analytical techniques.

For the 1980s, I believe that we will see a continuation of automation with a gradual improvement in the software and detectors, but nothing as revolutionary as the breakthroughs in the 1970s.

Inductively Coupled Plasma

The application of emission spectroscopy for elemental determination by the analytical laboratory has been popular for over 40 years. A simple way to excite atoms for emission spectroscopy is to burn the sample in a flame. Flame emission is largely restricted to those elements that are easily excited, such as the alkali earths, because of the relatively low temperature of flames (approximately 3000°C). A much higher temperature is needed to suitably excite elements such as Zn, Pb, Si, V, and Cd to achieve emission signals adequate for modern trace analysis.

Electric arcs or sparks are capable of producing the higher temperatures necessary for determination of elements which cannot be achieved with flames. Multielement determinations are possible with arc/spark spectroscopy through the use of direct reading spectrometers. Use of the arc/spark with a direct reader greatly increases the speed at which a sample can be analyzed for more than one element. Unfortunately, arc/spark spectroscopy is not a procedure that works well for a large variety of samples. It is especially useful for well characterized materials such as alloys, but it is difficult to use for samples of a variable matrix.

A relatively recent development in multielement analysis is the use of inductively coupled plasma (ICP). ICP offers many of the advantages of the arc/spark while eliminating many of the difficulties. The ICP is a hot discharge (approximately 10 000°K) which is a very efficient excitation source for emission spectroscopy, so that a wide range of elements can be excited. This makes the ICP ideally suited as a source for multielement analysis.

Attachment of an ICP to a direct reader, similar to those used in arc/spark spectroscopy, has created a very convenient instrument for multielement analysis. Commercial instruments (using direct readers or a scanning monochromator) which are available today, can determine in excess of 40 elements simultaneously.

Now that we have the ability to determine a large number of elements per sample, a problem of presenting the data in a usable form has been created. For example, if 25 elements are determined in 100 samples, then 2500 individual elemental determinations must be organized in a readily understood and readable manner. Such a collating problem can easily be handled by computer. Unfortunately, present commercial ICP data reduction systems do not allow for such organization.

A second problem of data management is simply "getting the numbers in the correct units." For example, in determining copper in a liver sample, the desired units are probably micrograms of copper per gram (μg/g) of liver. Currently, ICP data systems present the data as μg copper per mL diluted sample and the analyst must then transpose μg/mL values to μg/g by hand calculation. Some commercial ICP data systems present the data in units of μg/g, but these systems require that the same sample

weight and dilution factor be used for all samples, a situation that rarely exists.

Several "custom" data reduction systems have been developed to facilitate the calculation and compilation of data. These systems have been very beneficial to organizations analyzing a large number of samples. In most cases, however, the systems have been built around a second computer and hard copy facility, the cost of which can be excessive. The need for improved data reduction is an area that many manufacturers of ICP instrumentation should address in the future.

Gas-Liquid Chromatography/ High Pressure Liquid Chromatography

Gas-liquid chromatography (GLC) has developed over the past years in much the same way as NAA developed. In the 1960s, columns started with a crude liquid phase such as stopcock grease and developed through Carbowax to super Carbowax and to "polymer cages" such as Texex. At the same time, detectors were developing from general detectors based on thermal conductivity and flame ionization to more specialized detectors such as the nitrogen-phosphorus detectors and those based on flame photometry. There were also advances in temperature control, purer carrier gases, and more efficient designs. Finally, microprocessor controllers and data reduction facilities were developed.

An outgrowth and extension of liquid chromatography was high pressure liquid chromatography (HPLC). Improvements in columns and column packing combined with detectors which have ranged from the universal refractive index detectors through ultraviolet (UV) and variable UV fluorescence to electrochemical detectors have made this technique extremely useful. Again, microprocessor controllers for the pumps and automated data reduction capabilities combined with graphics have further advanced the technique.

In each instance the addition of microprocessors and automated techniques have increased sample throughput and aided in the interpretation of the results. In none of these techniques will the addition of automated features make a poor analyst good, nor is it a solution to all problems. However, it will make a good analyst better and improve sample throughput. Computers are only as good as the information going into them and as good as the software controlling the process. As software gets better, the same analyst also gets better and more efficient, and more information can be obtained reliably from one analysis.

The Analytical Method

Many analytical techniques are incorporating automated procedures as an integral part of the instrument. What will this mean to the collaborative study and to the AOAC?

An analytical method may be divided into the following steps:

(1) selection of a representative sample;

(2) preparation of the sample for determination by dissolution, etc.;

(3) determination of the element/compound in question;

(4) reduction of the data; and

(5) use of high quality known standards.

During the 1960s and into the 1970s, the skill of the analyst in each of these steps had a direct influence on the precision and accuracy of the analysis. With the introduction of automation, the determinative step was standardized. Late in the 1970s and into the 1980s the automated reduction of data became standard. The success of the analysis became a function of sample collection, preparation, and the adequacy of software controlling the automated portions of the analysis.

If we assume that the rules for obtaining a representative sample have been well defined, this leaves only the preparation of the sample for the determinative step as an analyst variable. In some cases even this step has been automated. This does not mean that the automated answers will be more correct, however; it only means that the analyst variability will be reduced.

During the 1980s we will see continuous improvements in the software controlling analyses. Thus several questions should be addressed relative to the AOAC standard methods. Does a change in software mean that the study should be redone? This would include any change in the determinative step or the data-reduction step. If the collaborative study was done using one company's automated system, would the study still be valid using a different company's automated system?

Finally, there are the standards to consider. For the past few years Standard Reference Materials (SRMs) such as flour, spinach, oyster, and bovine liver, which are certified for elemental content, have been made available by the National Bureau of Standards. There is still a need for organic standards in food matrices, as shown

in a recent conference on nutrient SRMs[1]. Many problems are involved in producing nutrient SRMs, but I believe that during the 1980s many of these will be resolved and SRMs will be available. Standards are an integral part of the analytical system because they allow checks on the methodology and procedures.

Upcoming Methodology

In each area there is usually one method which becomes the workhorse. I have listed several areas in which the FDA is currently working and the method(s) which workers in those areas feel will be the most useful.

Nutrients.—HPLC will be used extensively during this decade. Versatility and speed combined with automated features and improved columns ensure widespread applications for this technique.

Elemental Analysis.—Clearly, the workhorse for elemental analysis, both toxic and nutritional, will be ICP. Ability to determine 25 elements at one time and reduce the data almost on-line are very attractive features. If NAA is combined with ICP, the ability to determine about 40 elements including some overlap for checking purposes makes this a very attractive combination.

Fats and Oils.—A combination of GLC methods will be the preferred approach, with a trend toward capillary techniques combined with instrument automation.

Pesticides and Industrial Chemicals.—The use of capillary GLC will continue, and it will be developed from a qualitative into a quantitative technique. HPLC will receive widespread use as more selective detectors are developed.

Mycotoxins.—The area of mycotoxins will use a variety of techniques such as capillary GLC and HPLC with improved detectors and capillary chromatography. In addition, radioimmunoassay and enzyme-linked immunoassay will be important techniques. GLC–mass spectrometry will continue to be useful.

Conclusions

During the 1980s there will still be a push for lower and lower detection limits and for improved accuracy at low levels. Improvements will be made in each of the analytical fields but efforts will be concentrated on automated microprocessor-controlled and software-controlled instruments and data reduction to conserve analyst time and minimize analyst-to-analyst variability.

Acknowledgments

The author wishes to thank Henry Hollifield, Charles Thorpe, Martin Bueno, Ronald Suddendorf, Ted Chambers, and William Stroube, Food and Drug Administration, and Larry Doemeny, National Institute of Occupational Safety and Health, for their help in preparing this paper.

[1] Workshop on Reference Materials for Organic Nutrient Measurement, National Bureau of Standards, Oct. 23, 1980, Washington, DC 20234.

Analytical Methods for Microbiology in the Next Decade

JOHN FELDMAN
Food and Drug Administration, Minneapolis Center for Microbiological Investigations, Minneapolis, MN 55401

As microbiologists develop and modify methods and turn to automated equipment to decrease analysis time, the precision and accuracy of the methods must be ensured through collaborative study. Rapid, validated methods are needed in many areas. Sterility testing and the use of biological indicators are among subjects of interest to FDA and industry. These groups are also studying particulate analysis. The limulus amebocyte lysate test is being considered for use in detecting endotoxins in drugs and medical devices, in diagnosing illness, and in assessing the microbiological quality of foods and water. Reliable methods are needed for detecting viruses in foods to assure a safe food supply. Studies are needed on the Vibrionaceae to better understand the mechanisms of invasiveness and host response. Methods must also be developed to study pathogens in the water and food supply.

The 1980s should bring microbiologists a new challenge in method development, based on the success of the past decade. Our progress, however, has been slow and a bit disappointing compared to that of chemistry, for which instruments have been in use for some 20 years. Microbiology is largely a labor-intensive activity. This point is emphasized in the 75th Anniversary issue of the *FDA Consumer*, which contains a picture of bacteriologists in the old Bureau of Chemistry laboratories about 1912. The picture reveals analysts preparing tubes and tubes of media. The startling fact is that in our laboratory just last month, our analysts were still preparing tubes and tubes of media, only in a more modern facility and in a more efficient manner.

Progress has been slow. However, in that same laboratory our analysts were using gas chromatographs, computer terminals, API systems, and electrical impedance equipment. These instruments should lead to methods that make the work of the microbiologist easier. We must keep in mind that as we automate our methods we must maintain the precision and accuracy of manual methods. We must ensure this through the collaborative study approach that the AOAC and other scientific organizations have fostered for almost a century. The goal of the collaborative study is to ensure that methods will perform with the necessary accuracy and precision under usual laboratory conditions. These methods must be reliable for regulatory purposes. This approach has brought the validation of analytical methods to a high degree of perfection.

This discussion will cover a wide variety of areas currently of interest to the Food and Drug Administration (FDA) and industry, including sterility, particulate, pyrogen, pathogen, and sanitation testing. Any reference to specific equipment is only for illustration and does not represent an endorsement.

Sterility Testing – Biological Indicators

Sterility testing has been an official test in the *U.S. Pharmacopeia* (USP) for determining if products to be sterilized were, in fact, "sterile." The concept of sterilization and, consequently, the definition of "sterile" have been seriously questioned for the past decade by most experts in this field. The destruction kinetics or sterilization death time of microorganisms is now recognized as a probability function and subsequently leaves the old definition of sterile, which was "the absence of viable microorganisms", open to interpretation. In other words, when is a product considered to be sterile, at 10^{-2}, 10^{-4}, or 10^{-6} probability of survivors? The sterility test, although useful to determine gross contamination, is not adequate to determine the degree of sterilization of product. The adventitious contamination rate during sterility testing is generally considered to be one in a thousand. Thus the reliability of determining the degree of sterilization can never be greater than 10^{-3} and may be much less.

A more accurate determination of the probable degree of sterilization is being accomplished through the use of process control sterilization cycles. Process control provides the manufacturer with the data to determine if a desired sterilization process has been accomplished. Some of the parameters that must be known include the following: (1) The bioburden on the

This paper was presented at the Symposium on AOAC Methods Development—Challenge of the Next Decade, 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

0004-5756/82/6503-0535-07$01.00

product, that is, the number and type of microorganisms found. This in itself is useful information. However, the sterilization kinetics of the bioburden should be known.

(2) The penetration rate of the sterilant into the product, be it steam, dry heat, a gas such as ethylene oxide, or a liquid sterilant.

(3) The reliability of the equipment being used, such as the sterilizer itself, the gauges used, or the gas concentration detectors in the case of ethylene oxide.

(4) Physical parameters such as temperature, steam quality, relative humidity, and gas concentrations, all of which may affect the process.

(5) The effect of the sterilization process on biological indicators.

When all the necessary parameters are known for a sterilization process, a sterilizati n cycle can be designed to accomplish any degree of microbial destruction. When a sterilization process has proven to be sufficiently reliable, FDA may choose to allow "process release," or release without end product sterility testing. Process release or dosimetric release is being accomplished on a routine basis by many of the radiation sterilization facilities. More recently process release has been granted to certain facilities doing ethylene oxide sterilization.

Much of the process control is now being accomplished by computerized automated equipment. The equipment provides a means of precisely duplicating a sterilization process, and provides most of the quality assurance data.

The MSI (Microbial Survivor Index) is a concept being promoted by the Canadian government to indicate the degree of sterility of a product. The MSI reflects the probability of sterility; for example, a product that has undergone a sterilization process adequate to have a probability of a survival of less than 10^{-6} will have an MSI of 6.

There has been much discussion in this country about the effect of MSI, for example, on products used in a hospital. If a device having an MSI of 3 were used on a patient instead of one having an MSI of 6, what would be the liability of the user? If it were found to be necessary because of liability reasons for everything to have an MSI of 6, the cost of many products would be greatly increased. The MSI, however, would provide the user with more knowledge concerning the "sterility" of a product which, in turn, would allow him to make decisions concerning the use of that product. Other questions are whether a product sterilized to a 10^{-6} probability will truly maintain that probability and whether the MSI number should reflect the sterilization process or the probability of sterility when used.

Biological indicators (BIs) have been used to monitor a sterilization process for many years. They demonstrate the ability of a sterilization process to kill a certain number of resistant microorganisms, usually spores. The degree of reliability of BIs has been questioned in the past because of the variability encountered in this resistance. More recently, methods and equipment have been developed to produce uniform spore crops with known resistance and to provide the sterilization industry with a reliable tool for monitoring a sterilization process.

When the resistance of a BI to a sterilization process is known and the bioburden is known, a process can be designed to maximize the sterilization while minimizing the exposure of the product to the sterilant. New BIs are being developed for steam sterilization processes that use temperatures less than 118°C. This is necessary because *Bacillus stearothermophilus* is usually not killed below that temperature and consequently makes a poor indicator.

Particulates

The FDA became interested in determining numbers of particulates in large volume parenterals in the early 1960s, and in 1976 microbiologists from five laboratories in the field were trained to perform particulate analysis. Initial training consisted of microscopic detection and enumeration and equipment cleaning and use.

Part of the cleaning procedure entailed use of Freon as a final rinse. The suspected build-up of this highly volatile substance in the laboratory and especially in laminar flow clean benches posed a potential hazard to the analysts. Tests were made to determine if the Freon was necessary and ultimately showed that indeed it was not. Data were presented at the Parenteral Drug Association meeting to support the non-use of Freon and ultimately this was accepted by the USP. A change was made in the USP XIX, 2nd Supplement.

FDA has evaluated the microscope procedure for enumeration over the past 4 years to determine its repeatability and reliability. The method, as described in USP XX, will provide reliable data only when used by a highly trained analyst who does the work on a regular basis. The microscopic method was tedious and time-consuming and required total commitment by the analyst. The use of the automatic electronic

particle counter was investigated and was found to be much faster and to provide more repeatable counts. However, it was necessary to use the microscope to calibrate the equipment. A study by 4 laboratories comparing the electronic particle counter and the microscopic method revealed what one would expect. Those who did well with the microscopic method also did well with the automatic counter. The FDA is presently working on methodology to calibrate the automatic counter without relying on the microscope. The use of standard particles at first appears to be a logical approach to calibration; the difficulty lies with the numerous shapes and sizes of the particles to be enumerated and the type of electronic counters to be used. Some counters size particles by light blockage, some by light scattering, and some by displacement. Spherical particles of latex material may be used as a standard only if spherical particles are to be analyzed in the product; otherwise the enumeration of particles of various sizes will not be equivalent on various instruments because the instruments will see long thin particles differently depending on the detection system. For example, a long thin particle will be seen by a light blockage device as a surface area in reference to its orientation as it passes through a sensor, the displacement device will see the same particle as a volume, and the light scattering device will see it differently depending on the refractiveness and composition. All of the automatic counters will provide accurate data but they must be calibrated with a particle standard capable of simulating the types of particle to be counted.

Particle enumeration has been in the forefront of particle analysis, and identification of the particles is becoming more important. Some particles may not be dangerous even though they are present in a product, for example, a starch particle. On the other hand, a particle of asbestos, which has been shown to be a carcinogen, would be of much greater significance. Recent studies showing particulates derived from glucose in glucose solutions leave open to question the extent to which particle enumeration should be relied upon.

The particulates problem with small volume parenterals is somewhat similar in that the particle enumeration and identification need to be evaluated. It is unique, however, in the degree of particulate contamination and the source of the particles. Ampules, for instance, when broken open tend to contaminate the contents with glass particles. The type of glass and the way it is opened significantly affect the number and size of particulates formed.

Standards have not been set for small volume particulates primarily because the methods of particle analysis quantitatively lack the accuracy and reliability to provide the data base. FDA is working in the laboratory and on committees with industry to help solve some of the problems associated with particulate analysis and to help set standards of excellence in both industries.

Limulus Amebocyte Lysate

Limulus amebocyte lysate (LAL) is a biologically active fluid derived from the amebocytic blood cells of the horseshoe crab, *Limulus polyphemus*. The protein portion of the blood acts as a pro-clotting enzyme, and in the presence of a divalent cation such as a Ca^{2+} or Mg^{2+} and endotoxin, a clot is formed. In 1956 while doing research on the diseases of marine animals, F. B. Bang observed that the blood of a horseshoe crab clotted when it was injected with inactivated Gram-negative marine bacteria. In 1968 J. Levin and Bang determined that fluid from the ruptured amebocyte blood cells was the sensitive portion of the blood. Unfortunately, the crabs could only be bled at certain times of the year because of the production of an inhibitor in their blood. By 1972 S. Watson had succeeded in removing the inhibitors via chloroform extraction, thus allowing the production of LAL reagent at any time of the year. In 1977 the first commercial manufacturer was licensed by the Bureau of Biologics to produce LAL. In the following years 7 more manufacturers have been licensed and the sensitivity of the LAL test was increased from the nanogram to the picogram level. These and many more developments have aroused interest in using the LAL test for a variety of analyses ranging from the detection of endotoxin in drugs and medical devices to the diagnosis of Gram-negative meningitis or the determination of the microbiological quality of foods and water.

The impact of the LAL test on both the pharmaceutical and medical device industries has been considerable. It offers the elimination of the expense of housing and feeding rabbit colonies (it is only 1/15 to 1/30 as expensive), and results are available after a 1 h incubation period rather than the 3–8 h needed for the rabbit pyrogen test. Many drugs such as anesthetics and radiopharmaceuticals that previously could not be tested or that required considerable dilution to be tested because of their lethal effects on rabbits can now be tested for the presence of

endotoxin with the LAL test. Many manufacturers now perform the LAL test in parallel with the rabbit test to obtain data that will support the replacement of the rabbit test by the LAL test. One of the biggest problems facing the pharmaceutical industry with the use of the LAL test is overcoming inhibition problems that occur with some drugs (mostly small volume parenterals) and making them compatible with the LAL test. In the medical device industry perhaps a whole new approach can be used. No longer will devices have to be rinsed with 20–40 mL of liquid to obtain a sufficient amount to test; the LAL test only requires 0.1 mL per test. What is needed now is a procedure that is capable of recovering the very sticky endotoxin molecules from the surfaces of devices. Inhibition problems that may occur from substances being leached out of the devices during the rinsing procedure will have to be overcome.

The LAL test is also demonstrating that in the future it may play an important role in the clinical field. It has already been helpful in diagnosing several illnesses such as Gram-negative meningitis, mastitis, urinary infections, and endotoxemia. Its rapid detection of endotoxin (and therefore the sure knowledge that Gram-negative bacteria were or may still be present) could result in a patient receiving the correct treatment sooner than if a physician must wait 24–48 h for a bacterial culture to grow. It has proven useful when working with cerebrospinal fluids. A great deal of work is being done to adapt the LAL test to detect endotoxin in human blood. In the past its usefulness has been limited because of the presence of inhibitors in human blood but several methods are now being examined for their ability to overcome these inhibitors.

In the area of the microbiological quality of food and water the LAL test is again proving to be a valuable tool. Numerous comparisons have been made between the direct serial dilution method of detecting endotoxins and the Most Probable Number (MPN) methods in meats. It is possible that in the future, rather than the minimum wait of 48 h for a coliform MPN, the LAL test will be able to give a quantitative answer in 2 h and may prove to be more economical by eliminating the need for hundreds of tubes of media and incubator space in which to keep them.

It is clear that the LAL test is going to play an important part in the future of microbiology. The FDA is expected to issue new guidelines for replacing the rabbit pyrogen test with the LAL test for the end product testing and release of drugs and medical devices. With these guidelines a tremendous increase in the use of the LAL test for parenteral drugs is going to develop in the next 2–3 years. In order to switch from the rabbit to LAL test, manufacturers will only have to prove that their product is not inhibitory and through their quality assurance departments be able to demonstrate that their laboratories and personnel are qualified to do the test.

Pathogens and Viruses

According to the World Health Organization, laboratories in only 12 countries have research programs in food virology. Trained microbiologists capable of doing food virology are few. To address the problems of virus transmission through foods, increased communications and better coordination between researchers is needed. The most pressing need is for the development of reliable methods to detect viruses in foods. Until reliable and acceptable methods are developed, it will be difficult to assess the public health significance of such problems. In 1979 there were 460 reported outbreaks of food poisoning in the United States and in 37% of them etiology was confirmed (1). What about the other 63%? Were any, in reality, virus pathogens? Viral hepatitis is reported to account for 2.9% of the outbreaks and other virus-associated illness about 0.6%. Lack of data, due primarily to lack of reliable methods, makes assessment of the epidemiology, infectious dose, and contamination control recommendation difficult, and we need to better understand these in order to assure the consumer a safer food supply.

Humans are one of the greatest pollution sources through both excretions and secretions (2). Recycling domestic wastes to improve the environment, lower costs, and find suitable uses has encouraged the use of treated sewage as fertilizer. Studies show that the use of such fertilizer on vegetable crops presents hazards (3). Methods are needed to remove, or inactivate, heavy metals, parasites, and viruses in treated sewage.

The use of indicator organisms is another area that has caused controversy; however, further research is needed (4, 5). The indicator organism approach could result in low cost, rapid, and effective monitoring of the environment, particularly shellfish harvest waters.

Reports vary, but indicate that 1–20% of shellfish contain human viruses. Viruses have been detected in cow's and goat's milk; beef, pork, and fish products; processed meats; vege-

tables; and fruits. Future studies need to address the effects of processing and distribution on viruses in the final product. Current virus detection methods indicate that many foods contain some human viruses. Human viruses do not multiply in foods. Viruses require susceptible living cells and appropriate temperatures to multiply. These small accidental contaminants are very difficult to separate from high protein foods.

Some viruses do not replicate in the currently used tissue culture systems. It will be necessary to find additional susceptible cell-lines. Methods other than cell-line systems do provide alternatives. The scanning electron microscope is currently used to detect rotaviruses, a cause of infant diarrhea. Enzyme linked immunosorbent assay (ELISA) has been used extensively for bacteriological studies and may become equally important to virology.

The future will require time, equipment, laboratory space, trained microbiologists, and an interested and sympathetic management to solve the problems faced by the food virologist.

Vibrio

Vibrio cholerae non-01 was implicated in 5 cases of food poisoning in 1979 (1). Some people refer to these cholera organisms as non-agglutinable or non-cholera vibrios. Five cases seems quite a small number; they represent 0.1% of the total reported to the Centers for Disease Control (CDC). Yet 1979 was the first year that this illness was reported in the United States Does this mean we now have a new agent to look for or is it one that has been overlooked in the past? An outbreak of *V. cholerae* 01 was reported in 1978. (This illness was once thought to be an African and Asian exclusive.) These illnesses were associated with eating raw shellfish in the United States. The shellfish consumed were traced to harvest areas having elevated fecal coliform counts. The incidence of cholera poisonings will probably be kept low by the continued monitoring of harvest areas by the National Shellfish Sanitation Program.

Vibrio parahaemolyticus was implicated in 1.2% of the food poisoning outbreaks reported to CDC in 1979 (1). Very often more outbreaks occur in the summer, but 1979 was an exception; all outbreaks occurred in February and all shellfish were consumed at home with no deaths reported. All shellfish were cooked. This means that errors in refrigeration, cooking, and food handling hygiene were to blame. The small number of *V. parahaemolyticus* food poisonings shows that most food handling at the commercial level is adequate. Studies show that the organism is found in all U.S. coastal waters.

V. parahaemolyticus is a very important cause of illness in Japan, due primarily to the consumption of raw fish (6). In this country the freezing of seafood in storage and our habit of eating cooked fish have kept this potential food poisoning organism as a minor cause of illness. Its constant presence should not be disregarded, however.

The Vibrionaceae, considered here, are spread by contaminated water and food. It is the convalescent carrier or asymptomatic individual who pollutes the water supply. The illness is not spread by person-to-person contact as long as reasonable personal hygiene is maintained. It was found that 10^8–10^{10} organisms were required to produce illness in volunteers (7).

The present laboratory analytical methods are reliable but cumbersome. Better methods should aim at making analyses easier and faster and reducing costs. Not all microbiologists agree on what is the "best" method. There is a need to do collaborative studies and studies that will provide us with an understanding of the mechanisms involved in invasiveness and host response. By this means, better approaches to prevention, control, and patient care will be possible. This could involve adherence studies, plasmid mediation, and use of tissue culture in invasiveness studies or toxin production.

Pathogens/ Parasites

The increase in land application of domestic waste will increase the risk of exposure to many parasites. The disposal of waste through discharge to water systems provided an initial protection through dilution plus a period of natural purification before significant human contact was made with the polluted water downstream. This protective barrier does not exist with land application.

The occurrence of bacteria and viruses in wastewater, sludge, and receiving soils has captured the interest of many investigators. No such interest has been stimulated in the occurrence of the larger pathogenic organisms; few studies have included the study for protozoa and helminths. This is a significant omission because some of these organisms can survive waste treatment of land longer than any of the bacteria or viruses.

Secondary sewage treatment, including normal disinfection concentrations of chlorine and ozone, is ineffective in destroying parasites. In

fact, treatment conditions may encourage the embryonation of helminth eggs. The heavier eggs of some parasitic species, e.g., *Ascaris*, settle out during sewage treatment and appear in sludge, while the lighter cysts and eggs appear in the effluent. Most cysts and ova are relatively dense because the majority of the parasitic forms are found in the sludge.

The protozoans appear in sewage in cyst form. From the trophozoite stage in the intestinal tract, the organisms round up to form a precyst, and then secrete a tough membrane to become the cyst. These cysts are excreted in the feces.

The protozoan of greatest interest in recent years has been *Giardia lamblia*. As late as 1965 the pathogenicity of this organism was questioned. Renewed interest was stimulated by a large number of cases of giardiasis that occurred in Americans returning from the Soviet Union. In an Aspen, Colorado, outbreak, cysts were detected in 2 of 3 sewage lines serving the town. In 1979, an outbreak of foodborne giardiasis appears to be the first documented case showing fecally contaminated food to be a vehicle for the transmission of giardiasis, even though the fecal–oral route of transmission has been well established.

At least 15 different parasitic helminths may be capable of producing a health problem from sewage exposure. None of the intestinal nematodes requires an intermediate host and most produce prodigious numbers of eggs. The eggs, particularly those of *Ascaris* and *Trichuris*, are very resistant to environmental effects.

The presence of protozoans and helminths as mentioned earlier provides us with an excellent reason for wanting to control or monitor the use of sewage on agricultural lands. To accomplish this, methods must be developed that can quantitatively check the sewage placed on fields and can sample crops grown on these particular fields for the presence of parasites.

Ascaris eggs are the most resistant of all the sewage pathogens to treatment and environmental stresses. Therefore, *Ascaris* would appear to be the limiting organism in determining the health risk from sewage-treated soils. If detectable numbers were present in raw sewage, the quantification of *Ascaris* eggs in treated sludge or soil could possibly serve as an index of the potential health risk of infection from any pathogen originally present in the sewage. Methods for the recovery of *Ascaris* eggs must be collaborated until a fast and efficient method is found which can be used with confidence.

It is going to become a big problem in the future when stricter laws are put into effect for regulating the pollution of our rivers and streams. Some people suggest using only pasture land for sewage disposal. All this may do is delay the inevitable. Cattle, sheep, and other animals used for human food can pick up different parasites and eventually pass them on to the consumer. We must have control over the sewage being used on the land and how the land is used. Fifty percent of our sewage is currently disposed of in various land application programs and the amount is continually increasing. Now is the time to develop methodology required to ensure the health of the consumer.

Sanitation

At the beginning, I alluded to the fact that our laboratory was still preparing racks and racks of tubes which will be predominantly used for the detection of *Escherichia coli* and the coliform bacteria. This analysis is conducted in conjunction with the determination of *Staphylococci* and aerobic plate counts in an effort to establish microbial criteria for food.

This analysis can be very costly. The spiral plating instrument has been successfully collaborated for use in the aerobic plate count area. Although it costs about $6,000–$7,000, the savings in petri dishes and media may well make up this initial outlay. Other techniques that could be used for plate count are bioluminescence and the radiometric method; however, the sensitivity of the procedures at present leaves something to be desired. With some modification of the dilutions, the spiral plating equipment may be adaptable to *E. coli* or *Staphylococcus* organisms. This will naturally require more selective media in concert with rapid biochemical identification methods. In summary, the entire area of sanitation microbiology may lend itself to some form of automation.

In conclusion, although we have made considerable strides in the development of microbiology methods in the past decade, we still have quite a challenge before us.

REFERENCES

(1) Department of Health and Human Services (1981) *Foodborne Disease Surveillance Annual Summary 1979*. Public Health Service, Centers for Disease Control
(2) Larkin, E. P. (1981) *J. Food Prot.* **44**, 320–325
(3) Larkin, E. P., et al. (1978) "Land application of sewage wastes: Potential for concentration of foodstuffs and agricultural soils by viruses, bacterial pathogens, and parasites." In *State of Knowledge*

in Land Treatment of Wastewater, Vol. 2, International Symposium, H. L. McKim (Symp. Coord.), Aug. 20–25, Hanover, NH, pp. 215–223
(4) Berg, G. (Ed.) (1978) *Indicators of Viruses in Water and Food*, Ann Arbor Science Publishers, Ann Arbor, MI
(5) Irving, L. G., & Smith, F. A. (1981) *Appl. Environ. Microbiol.* **41**, 51–59
(6) Fujino, T., Sakaguchi, G., Sakayaki, R., & Takeda, Y. (Eds.) (1974) International Symposium on *Vibrio parahaemolyticus*, Saikon Publishing Co., Tokyo, Japan, pp. 148–149
(7) Davis, B. D., et al. (Eds.) (1980) *Microbiology*, 3rd Ed., Harper and Row, New York, NY, p. 667

Analytical Methods for Drugs in the Next Decade

JOHN E. ZAREMBO
Revlon Health Care Group, Research and Development Division, Tuckahoe, NY 10707

Today's research for new drug substances is moving toward molecules of much larger molecular weight and complexity than the small, modern drug molecules, e.g., polypeptides, interferon, slow-releasing substances, and blood and plasma components. The complexity of natural products derived from human or other animal bodies requires a new analytical chemistry. Simple tests for drug stability, dissolution, and bioavailability will no longer suffice to provide and ensure purity and structural identity of such drugs. During the next decade, the demands on analytical chemists will increase; more biological organic and physical chemistry will be required by analysts to perform their jobs. Criteria for purity will require even greater chromatographic involvement but this will have to be complemented by nuclear magnetic resonance and mass spectrometry. The demands made on AOAC to validate and approve methods to assay drugs and determine their purity will increase enormously in this next decade. The understanding of drug purity will take on a new meaning because of the complex nature of natural products. Stability indication will be significant only when chemical and biological tests can complement and define the active components and the impurities. AOAC will be required to play a major role in assuring the manufacturer and the public that their drugs are adequately tested, safe, and efficacious.

Since the enactment of the Delaney Clause and the good laboratory and good manufacturing requirements of the Food, Drug, and Cosmetic Act, drug consumers have become newly aware of the serious and possibly adverse effects and potentially lethal dangers of poorly manufactured, impure, and unstable drugs. This realization has had a profound impact and has increased demands that information about any potential hazards be made known by regulatory officers who review applications to test and market new drugs and who insist that all such information be included in the applications. The result of this public awareness and demand has influenced the research and development philosophy of drug manufacturers and research scientists, and has had a marked effect on drug research, quality control, and quality assurance.

Drug purity and drug safety would seem to be equivalent terms. It might seem logical that the purer the drug substance and dosage form, the safer the drug. However, this can be determined only through adequate safety tests on animals and humans. In addition, drug potency has been equated with stability, availability, and efficacy. This is certainly an important consideration because the consumer is the final test of drug efficacy and safety.

These concerns will continue and intensify during the 1980's as more sophisticated instrumentation and diagnostic test methods are introduced and as we learn more about the long-range effects of drugs, especially on chronically treated individuals. Most of us will eventually suffer the pain and diseases of age and aging, and it will become important to us to have drugs to help us cope as painlessly as possible with the increasing liabilities that old age brings to our lives. Because of our demographic trend toward an older and longer surviving population, we are amassing data by which we can determine the long-range effects of our wonder-working drugs. Examples are osteoporosis, the development of carcinomas, and mutagenic and teratogenic effects, which do not seem evident in the early or limited stages of drug safety testing and research. Many of these adverse effects surface only after many years, even after therapy or exposure has terminated. Much of the information that can be correlated with adverse drug effects and that we need to help overcome these problems will be derived from the sophisticated determinations of purity, stability, and integrity as well as the kinetics and mechanisms of chemical change (which may be detrimental or even lethal) made in the analytical laboratory.

Currently, one of the major assays used in drug research is the stability assay, which assures the consumer that the active component in the dosage form will dissolve and be available so that the body's biological system can do its job. This test also helps to ensure the quality and safety of the drug. It permits us to deal effectively with the problems of establishing the composition of drugs all through their shelf-life, during devel-

This paper was presented at the Symposium on AOAC Methods Development—Challenge of the Next Decade, 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

0004-5756/82/6503-0542-09$01.00

opment as well as during use. For such studies and for drug surveillance, the assays used must necessarily be stability-indicating.

A stability-indicating assay, therefore, not only must be able to determine the potency of the intact drug in the dosage form but, equally as important, must be able to detect and quantitatively determine most of the decomposition products, especially any potentially dangerous ones. If there is any possibility that these decomposition products can negate the assay results, it is imperative that the analyst correct for them. Equally important, the analytical stability measurements must be able to determine the occurrence of crystalline changes, such as polymorphic modification, hydration, or solvation, which can change the availability and hence the rate of dissolution and efficacy of the dosage form.

An assay of this type is quite complex because of the multitude of components making up a drug preparation. In addition to the active substance, a drug may contain excipients, solvents, water, lubricants, dispersing agents, colorants, and flavors, etc. Certain drug formulations may contain as many as 40 or 50 different substances. The methodology used to obtain meaningful and accurate data requires considerable sample preparation, such as extractions and isolations of the components being sought, without alteration of the true drug composition. This is a formidable assignment.

The determination of the physical changes, such as polymorphism, requires other approaches, since polymorphism is destroyed when a drug is dissolved; solvation and hydration are also affected by the sample preparation process and might produce fictitious data. We therefore have to resort to methods that do not decompose or alter the physical condition of the drug substance; today's analytical sophistication is permitting us to do that.

Polymorphism, for example, can now be determined by a variety of techniques, such as x-ray powder diffraction, infrared analysis, differential thermal analysis, and chemical microscopy. From the new technique of solid-state cross-polarization nuclear magnetic resonance, we are learning that different crystalline and polymorphic forms exhibit different features that are easily detectable and in some cases can be quantitated. For the other aspects of drug integrity and purity, new detectors of greater sensitivity and specificity are either available or being developed. Examples are liquid chromatography with its multitude of approaches and new detectors, and field desorption, MS/MS, and fast atom bombardment mass spectrometry. Ion chromatography also appears promising for determination of inorganic anions and cations and some organic molecules and contaminants. In some cases it is possible to detect and quantitate compounds at picogram levels.

The validation of methodology for drug stability assays promises a major role for AOAC. There is the need to establish that current and future drug methodology is indeed stability-indicating and does produce accurate results for drug purity. In many cases thin layer chromatography has been used in combination with liquid chromatography, and this approach to fingerprinting and profiling drugs has proved to be invaluable. A neutral referee body is needed to critique and test many of these methods to determine their suitability and value. AOAC could play a major role in initiating, coordinating, and directing such studies. Such information would enable us to handle the analytical problems of drug research and development much more effectively in the 1980's.

During the next decade we can anticipate the introduction of many new organic molecules of higher molecular weight and greater complexity. We are on the threshold of a family of drugs that treat not only the major killers such as cancer and heart disease but also many of the genetic diseases. At the last count, more than 2000 such diseases have been described.

New drug substances are now emerging not only from organic synthetic and natural product laboratories but also from computerized automatic molecule synthesizers and most especially from genetic engineering and biological laboratories. They are being isolated from human plasma, blood, and tissue fractions, from aquatic animals, and from isolates of the entire animal, vegetable, and mineral world around us. The number is uncountable. Molecules such as human and animal insulins, gamma globulins, hormonal factors, human and animal calcitonin, leukotrienes, slow-releasing substances, antibiotics, interferons, prostaglandins, blood components like Factor VIII and Factor IX, polypeptides, oligosaccharides, and enzymes are merely a few. Each year brings hundreds, if not thousands, of new therapeutically and biologically active molecules. These molecules need to be characterized chemically and biologically. Their stability and integrity will have to be determined not only on the compound or mixture itself but also on the final dosage form at manufacture and during its shelf-life.

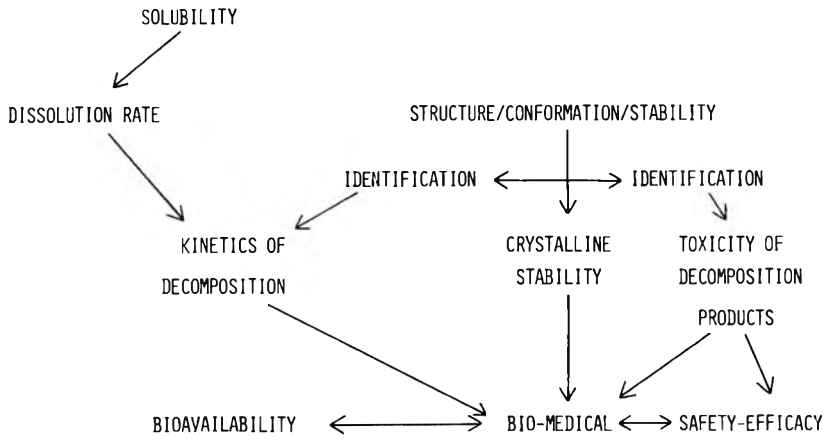


Figure 1. Preliminary information required for determining safety and efficacy of new drugs.

Figure 1 outlines the preliminary information required for determining the safety and efficacy that is eventually filed in a request for an Investigational New Drug (IND) and subsequently for a New Drug Application (NDA). These data are required not only for the regulatory agency but also for each of the research staff. This information helps to set the standards for drug adequacy, purity, chemical and biological integrity, and therapeutic efficiency, and gives a more complete understanding of the drug. We begin analytically with structure, conformation, methods of analysis, and stability. We also determine solubility, dissolution rate, kinetics of decomposition, and bioavailability in the blood stream. From solubility data we can predict the time for onset of drug action and determine whether the drug will partition from the gastric milieu and be available for absorption into the blood stream. Dissolution rate, one of the major factors in drug analysis and quality control, helps in evaluating the quality of the dosage form throughout its complete shelf-life. The kinetics of decomposition and the decomposition products provide information on drug action in the body from the time the drug comes in contact with highly acidic stomach fluids until it is dissolved, absorbed, and produces its action in the biological system in the presence of a variety of enzymes and other chemicals. Identification of what is happening becomes a major analytical and biological undertaking. The scientist is always concerned with what is happening to the molecule as well as to the consumer as chemical changes occur. This must be determined completely, and we always need to assure ourselves that the chemical environment from all this reactivity is safe and that the chemical changes occurring do not yield chemical species that will produce adverse effects. The question of toxicity and the amounts of potentially toxic material formed must be determined analytically. Since we cannot completely equate laboratory animal toxicity with human toxicity, the critical analysis and drug safety must be determined by human tests and the necessary analytical information must be derived from them.

Physical changes are equally important. It is possible to convert a soluble drug into an insoluble concretous mass that simply passes through the body and is completely ineffective. It is indeed important to follow drug integrity not only in the body but also outside it to establish that toxic impurities are not formed, that drug potency is maintained, and that the drug will dissolve and be completely available to the patient.

Figure 2 lists several of the analytical techniques currently used in profiling and identifying impurities. Some of the present and emerging techniques mentioned here will lighten the job ahead of us. Infrared spectroscopy, combined with Fourier transform and computer capability using enhancement and subtraction techniques, provides spectral identification in complex mixtures and at nanogram levels. The technique is useful for both quantitative and qualitative work. Polymorphism, for example, can be determined not only on a pure compound but even in the presence of excipients and other active components in the final dosage form. The formation of hydrates, solvates, and chelates can be determined by infrared techniques. Chemical changes, such as oxida-

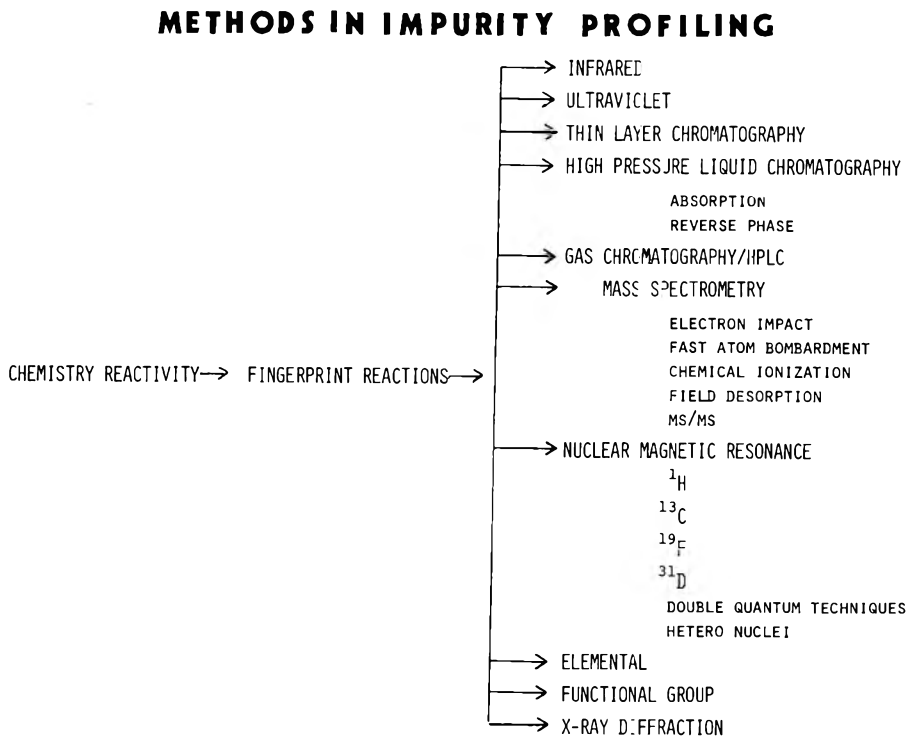


Figure 2. Methods in impurity profiling.

tion, reduction, isomerization, hydrolysis, and polymerization, can also be monitored by infrared spectroscopy.

In this past decade, high pressure liquid chromatography (HPLC) has become one of the most valuable tools in drug analysis and research. It is used for drug assays, for determining impurities in drugs, and for determining chemical instability of drugs. Much of our energy now and in the next decade will be devoted to refining this valuable technique into a completely automated, machine-controlled, and integrated system. With complementary instrumentation to do drug analysis, HPLC will probably provide the bulk of information needed to determine drug integrity and drug chemistry in routine use. We anticipate greater speed, resolution, sensitivity, and specificity. In conjunction with double quantum NMR techniques, NMR will permit us to look at molecules which contain two carbon-13 atoms; it will provide a new capability to determine the structure of molecules in solution which will rival that of single-crystal x-ray diffraction analysis.

In the 1980's we can expect dramatic, new NMR capabilities and uses in both drug analysis and drug research. We can expect more use of NMR with biological and complex molecules of high molecular weights. Sensitivity now is in the picogram range for protons and in the milligram range for carbon-13. Information on chemical structure of molecules is being provided most appropriately by NMR and mass spectrometry and by combined techniques. High resolution NMR with multi-nuclei capability and solid-state NMR are making structure determination less complicated. Complex spectra can be reduced to first order spectra to permit easier interpretation of data. Using telephone-computer connections, we are getting longer and improved instrument operation, quicker maintenance, and greater reliability. High resolution instruments (greater than 250 MHz) provide high sensitivity and require only a minimum of sample and a minimum number of transients. Samples can be analyzed just about as quickly as they can be prepared, and the computer allows excellent quantitation. NMR is both a qualitative and a highly specific and precise quantitative technique. Answers obtained by NMR are unequivocal, unlike those from chromatographic techniques, which have

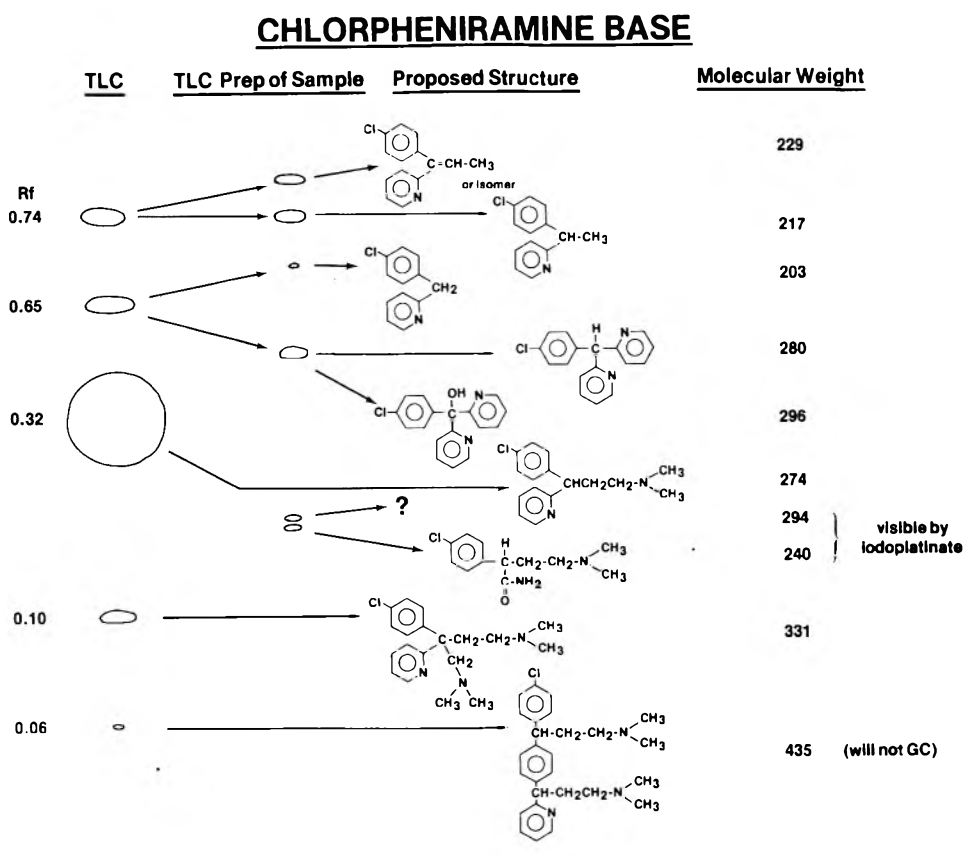


Figure 3. Typical analytical study to isolate, identify, and quantitate a drug and its impurities.

no real specificity because they depend on retention time alone as the identifying parameter.

Probably the most exciting new area of analytical research and analytical power is mass spectrometry; considerable progress is being made. Three mass spectrometric techniques, electron ionization impact, field desorption, and chemical ionization, allow us to look at large molecular weight molecules, up to approximately 1000 mass units. Field desorption essentially provides one molecular ion peak plus its isotope peaks, i.e., a cluster of peaks, for each compound present, so that it is possible to determine if impurities are present. The spectrum is uncomplicated. As a soft ionization technique, it does not break up the molecule into a peak for each possible mass up to the molecular ion. The next decade will see the development and expansion of negative ion mass spectrometry, tandem mass spectrometry (MS/MS), and secondary ion mass spectrometry (SIMS). SIMS sputters sample particles from a solid target surface; these ejected particles are then mass analyzed. Lower molecular weight nonvolatile compounds may thus be successfully analyzed.

The laser desorption technique, which has not been fully developed, appears to have promise for the analysis of nonvolatile molecules that now appear to be intractable. One of the most promising mass spectral techniques is fast atom bombardment. It may be a valuable tool for looking at the large peptide molecules we expect to encounter in drug research. At present, laser desorption can be used to inspect and examine molecules with molecular weights of 4000–6000. Work in this area at some universities suggests that compounds with molecular weights up to 15,000 or more produce molecular ions. Laser desorption appears to be one of the better ways in which we can study these high molecular weight materials, for example, interferon, slow-releasing substances, fragments of hPTH, and small enzymes. When coupled with x-ray

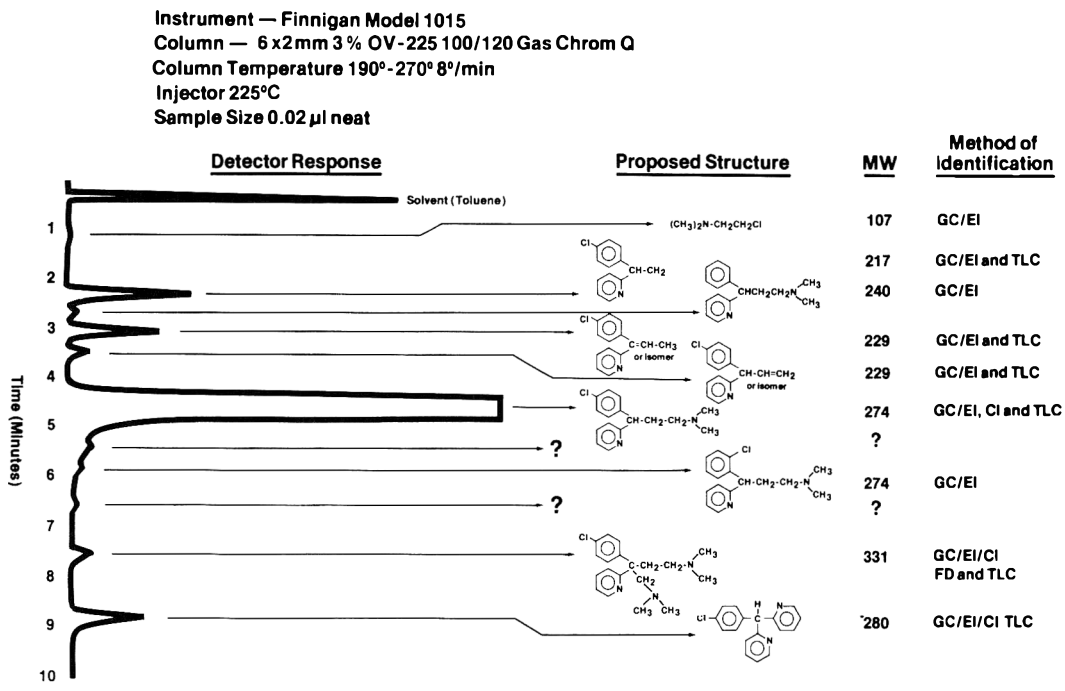


Figure 4. Impurity profiling using gas chromatography and mass spectrometry.

single-crystal analysis MS enables us to study the integrity of these structures and the subtle chemical changes they undergo that have a profound effect on their therapeutic efficacy.

Another area of considerable interest and value to the drug industry is the use of radiopharmaceuticals. Early in a drug study, information is needed on the bioavailability of the new drug substance, the body areas in which it accumulates, and the metabolic changes it undergoes. Radiolabeled drugs can be used to study questions of whether the molecule associates with other molecules or tissues and whether new species are formed which are then therapeutically active. Analytical technology is required to solve these problems. By coupling radiochemical detectors with HPLC, MS, IR, NMR, Fourier transform MS, and modern data systems, we can monitor and establish the structure, excretion, and disposition of these components in the body organs and body fluids. This helps us to establish their safety and efficacy as well as the mechanisms and kinetics of drug action. The new radiochemical detectors with their high degree of sensitivity are opening new capabilities for the 1980's. Even extremely small amounts of highly active drugs can now be studied and their action can be understood. The use, study, and validation of radiochemicals can present new possibilities for AOAC to design procedures to validate analytical methodology.

One other area will still be of great concern during the 1980's: the problem of impurities present in commercial drugs. This very real concern is often either ignored or only casually investigated. We are learning more and more of the toxic effects of extremely low levels of drug after long, continuous exposure. This is why drug purity and integrity must be studied and understood in light of the advances in drug preparation. Penicillin contamination can cause serious problems and even life-threatening effects in sensitive individuals. *N*-Nitrosamines at a few parts per million can produce hepatic cancer in dogs; many drugs and their nitrosated products may be a real concern for safety in humans. Another hazardous chemical class is the symmetrical tetrachlorodioxins, which may be formed when chloroaromatics, frequently used as intermediates in drug manufacture, are converted to dioxins under the appropriate synthetic conditions. Symmetrical tetrachlorodioxin is probably the most toxic small molecule known; it produces death at parts per million levels and

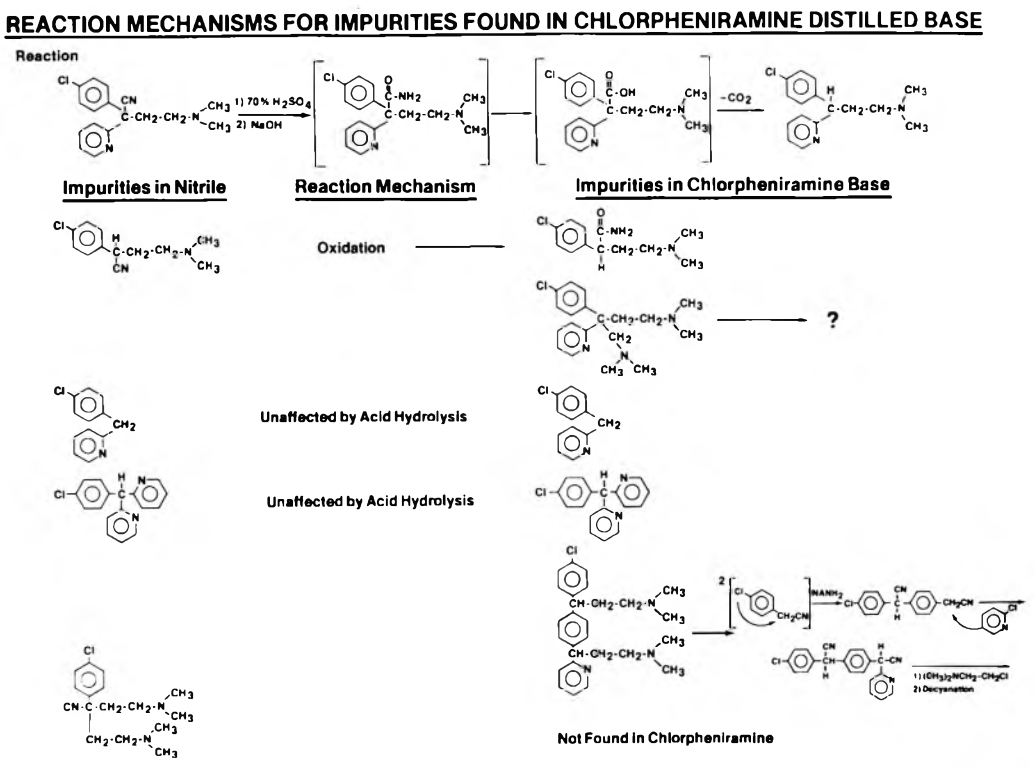


Figure 5. Determination of origin of impurities in a drug.

even less. A chemically modified H_2 antagonist that may be present in drugs at a level of 0.01% not only can cancel the therapeutic effect of the drug but can even produce the exact opposite and aggravating effect.

Besides these potential dangers, there is another issue regarding impurities in drugs: the case in which there are no dangers or pharmaceutical liability, yet excessive and unnecessary demands are made on the pharmaceutical industry. The pharmaceutical industry recognizes the problem of drug impurities and the need to control their presence. There is a need, however, for guidelines on permissible impurity limits so that excessive and unnecessary analytical time, manufacturing, and purification steps are not required that will increase manufacturing costs, result in higher drug prices, and even delay the approval of much needed therapeutic agents. Where the LD_{50} and other drug safety tests show no dangerous or adverse effects, a guideline should be established and the regulatory agency and the manufacturer should reach a general consensus.

Figure 3 demonstrates the thoroughness and completeness (and cost) of an analytical study of a common antihistaminic drug, chlorpheniramine, which is present in many over-the-counter and ethical drug preparations. This work required many isolations and the use of a combination of analytical techniques and instruments. Mass spectrometry and NMR were employed throughout. The study was carried out to demonstrate the type of information on impurities in drugs that can be obtained in a major effort to establish drug safety. Figure 3 shows the TLC profile of the drug followed by preparative TLC; the number of components increases as more material is spotted. Thus each impurity itself contains "impurities." Theoretically, the number of impurities in a compound could be so large that such a study could go on ad infinitum. Imagine the amount of effort and cost required to isolate, identify, and quantitate each impurity and to establish its structure and molecular weight.

Figure 4 shows a typical example of impurity profiling using GC and MS. Several of the MS techniques mentioned earlier were used, viz., electron ionization impact, chemical ionization,

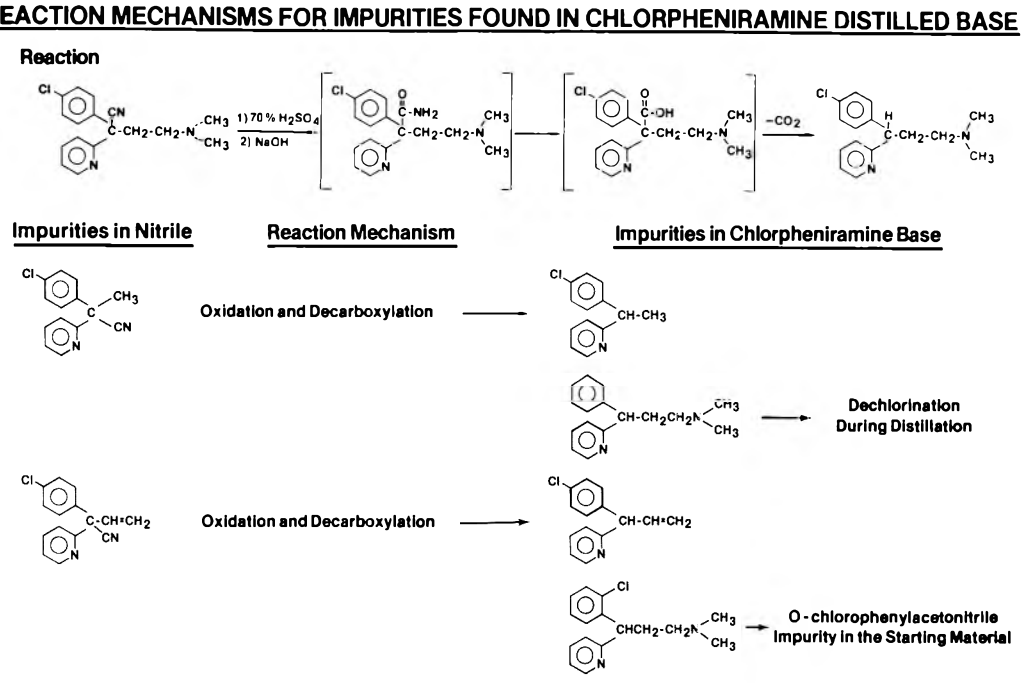


Figure 6. Additional steps in chemical drug analysis.

and field desorption. Final identification in some cases required TLC and/or preparative isolations and the use of NMR. This multitude of instrumentation and technology (over $1 million worth) was required to resolve this problem because no single method ever provides all the required information. Certainly this problem could be handled only by a highly sophisticated scientific staff with an array of instruments.

Figure 5 demonstrates the steps taken, once structures are established. We now wish to determine the origin of these impurities and determine how we might prevent their formation or remove them. Oxidation and hydrolysis are common sources of difficulty. Some of the impurities result from earlier synthetic steps and are carried through to the final product, thereby increasing the number of different impurities present because of secondary reactions.

Figure 6 provides an extension of some of the chemistry mentioned earlier. Oxidation and combined oxidation and decarboxylation followed by dechlorination during the distillation step explain some of the origin of these impurities. Impurities seem to arise from every possible chemical/physical reaction process, such as heat, light, oxidation, and hydrolysis. Such complexity in chemical drug analysis accounts for some of the high cost of drug development.

During the 1980's we can therefore look for greater use of computers to speed this process without diminishing the thoroughness that ensures the safety of our drugs and prevents the tragedy of adverse drug effects such as the more than 60 drug-related deaths due to selacryn (tricrynafen) reported in the press. Analytical instruments during the 1980's will become the accessories of the computer rather than the reverse situation that exists today. Full automation and even the use of robots to carry out repetitive, routine, and dangerous or complicated analyses is a reality. This should relieve the burden and ennui of routine work, improve precision and accuracy, and enable many more analyses to be carried out in much less time.

We face one major problem that the computer could help solve: the problem of data handling, storage, and management. Final reports of work and recommendations to rework, reject, or release could be prepared automatically. At the same time the possibility of omission, operator error, transcription error, and delays could be reduced and eliminated.

Another area of concern is the development of rapid methods and overnight or unattended

instrument methods using computerized and multisolvent and variable programmable steps. Such methods not only can be developed but also can be optimized, validated, and statistically evaluated without operator intervention, once they are set up. In the past few years AOAC has provided a forum for evaluating methodology, setting standards for performance, and enlightening the analytical community about progress and requirements in our scientific discipline. In the 1980's, AOAC should play a major role in the interface of industry, academia, the compendia, and regulatory agencies to prepare adequate, critical, and well tested and documented methods and standards for the analysis of our food and drug products.

The Next Decade: Sound Methodology and Legal Actions

ARTHUR N. LEVINE
U.S. Department of Health and Human Services, Office of the General Counsel, Rockville, MD 20857

Regulatory agencies become involved in numerous "legal actions." Not only do these agencies initiate legal actions to enforce laws, they promulgate rules, issue statements of policy, and develop and define statutory standards. Not surprisingly, these programs are challenged in courts by members of regulated industry, trade associations, consumer groups, and individual citizens. Regulatory agencies also initiate and respond to a variety of issues by administrative, rather than judicial, action. These too are "legal actions" and frequently concern the adequacy of laboratory and scientific methodology. Where the agency initiates action to enforce the law, it carries the burden of proof. Where agency action or inaction is challenged, the challenging party carries the burden of proof and the courts have frequently given great deference to the views of the agency. However, the courts will frequently evaluate for themselves the scientific basis for the agency's decision, regulation, or program. Examples of judicial reaction to scientific questions in recent years will be discussed.

I would like to identify five forms of "legal action."

(1) One form is rulemaking. This is the process by which a regulatory agency makes law by proposing a rule, soliciting comments, and then promulgating a final order, which is codified in the *Code of Federal Regulations*. Rules in the *Code of Federal Regulations* have the force and effect of law; they are binding and those subject to them must comply. When an agency wants to make a rule that is based on some assumption about scientific reality, the scientific data and the methodology to support that rule must exist and be carefully articulated.

(2) A second form of "legal action" is called "administrative adjudication;" however, it is not decided (initially) in a court, but rather between the proponents of some product or viewpoint and the regulatory agency. The decision to approve or not approve a New Drug Application (or a supplement) is an administrative adjudication. The agency acts like a judge. Its decision, the adjudication, directly affects only the proponent. The impact of such a decision is usually individual and contemporary; the impact of rulemaking, however, is general and prospective because it affects the future conduct of everyone. Here too, regulatory agencies rely on scientists to provide sound data, either to support the agency's position or question a proponent's data or analysis.

(3) Another form of "legal action" is legislation. Every year the Department of Health and Human Services and other executive branch agencies propose that Congress amend one or more of the statutes it enforces. Obviously, chances of obtaining the desired amendment exist only when there is valid scientific support for the proposition or program proposed to be enacted into law.

(4) The forms of "legal action" with which we are most familiar, and think of immediately, are those brought before federal judicial courts to enforce the law—seizure of products, proposed injunctions of firms, prosecutions of persons.

(5) Finally, there are legal actions in which the Department or the agency is the defendant. These are suits against the government. Some groups sue the government claiming that the government doesn't do enough. Other groups sue the government claiming that the government does too much. Most of these suits challenge agency policies, programs, and regulations. The agency promulgates a rule and someone comes into court and says, "That's a bad rule. It won't stand careful scrutiny." The agency, in its defense, will rely on the scientific data in what is called the "administrative record" to support the rule. Thus, rulemaking and defensive litigation are frequently two sides of the same coin.

The interaction of science with the courts is not new. One benchmark in the modern relationship between judges and scientists dates from the 1930's: a case involving a child labor law passed by the State of Oregon. The statute was challenged by persons who argued that the State had no right to dictate how much a child could work, and claimed that the Oregon statute prohibiting excessive child labor was unconstitutional. The State of Oregon hired a lawyer to represent it, and that lawyer hired an assistant. The case went to the Supreme Court. In those days, states

Presented at the Symposium on AOAC Methods Development—Challenge of the Next Decade, 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

0004-5756/82/6503-0551-04$01.00

could pass laws if the laws were reasonable; that was the legal standard. The brief that the lawyers for the State of Oregon filed in the Supreme Court had 2 parts. The first consisted of a statement that a state can do that which is reasonable. The second section of the legal brief contained, in essence, one sentence: "The State of Oregon has acted reasonably." And then, a footnote, of many pages, went on to cite sociological studies, medical treatises, psychological studies, and other statistical and scientific data showing that excessive work was detrimental to children. No one on the Supreme Court was a sociologist, psychologist, or pediatrician. Nevertheless, the Court undertook to review those references to the scientific literature and decided on that basis that the State of Oregon *had* acted reasonably. This kind of legal brief came to be named the Brandeis Brief, after the primary lawyer for the State of Oregon, Louis D. Brandeis, later to become a justice of the United States Supreme Court, as did his assistant, Felix Frankfurter.

I have been asked to speculate on the relationship between sound methodology and legal actions in the next decade. I would like to start with a disclaimer. I am not talking about the physical sciences, but about social science. When we enter the realm of social science, only social scientists believe that they can predict the future. Lawyers know better. There are several reasons why it is hard to anticipate what is going to happen in the next decade in the relationship between sound methodology and legal actions.

First, that relationship is largely predicated on the courts, and the personnel of the courts is currently very much in flux. A statute passed during the Carter administration significantly increased the number of federal judges. The perspectives of these new judges are substantially unknown. President Reagan has begun to appoint other new federal judges. As a result, the judicial arena in which science is evaluated is changing noticeably.

Second, the Supreme Court, the court of last appeal, is also in a state of flux, as we are all well aware. The Court has just installed one new member and according to the news media, there may be 3 to 5 new members within the next few years. What those justices do will have a very significant effect on the relationship between science and law.

Another factor is the United States Attorneys in each of the 94 federal judicial districts throughout the United States. When we talk of FDA judicial actions, we are talking about a legal action that occurs only with the approval of the local U.S. Attorney. FDA does not have the power to file cases; only the Department of Justice can file FDA cases, and it does so through its primary field agents, United States Attorneys. They report to the Attorney General of the United States. The views of these U.S. Attorneys very much determine what kinds of cases are going to be presented to the courts and with how much vigor. One of the reasons it is difficult to predict the '80s is that there are about 90 new U.S. Attorneys.

Another factor adding some uncertainty is the impact of the new era of federalism. Executive branch agencies are beginning to cope with reduced resources. During this period it will be hard to foretell how the relationship between science and law may be altered.

Finally, there is a bill currently pending before Congress which would give Congress a legislative veto over the rules promulgated by regulatory agencies. Should that bill pass in one form or another it will significantly affect an agency's independence in determining what is good science.

Those are some of the factors which we have to keep in mind as we speculate about the '80s and the relationship between sound methodology and legal actions.

As a lawyer, I deal in rules. I would like to discuss the rules that the courts apply to the different kinds of regulatory actions I have identified.

When an agency makes a rule, and that rule is challenged, courts ask the following question: Was the rule arbitrary or capricious? If it wasn't arbitrary or capricious, it stands; if it was, it falls. A few passages from a recently decided case involving EPA regulations for smelters should provide a sense of what arbitrary and capricious means.

"The reviewing court [the court reviewing the regulation] is required to engage in a "substantial inquiry," i.e., a thorough, probing, in-depth review. To determine whether the decision was arbitrary or capricious, the court must consider whether the decision was "based on a consideration of the relevant factors and whether there has been a clear error in judgment." *Id.* Although the scope of inquiry is to be "searching and careful" the ultimate standard of review is narrow. "The court is not empowered to substitute its judgment for that of the agency." . . . A satisfactory explanation of agency action is essential for adequate judicial review, because the focus of judicial review is not on the wisdom of the agency's decision, but on whether the

process employed by the agency to reach its decision took into consideration all the relevant factors. . . . The focal point of judicial review is the administrative record already in existence, "not some new record made initially in the reviewing court." . . . The EPA must provide a reasoned basis for its actions, fully explaining its course of conduct, i.e., that is its reasoning, its analysis, and its inquiry."

What do we glean from that passage? Courts do not (are not supposed to) consider whether the agency decided the issue the same as the court. They ask: What was the issue? What are the factors that must be considered in deciding the issue? Did the agency look at all the factors? The courts do not decide the case based on what the judges believe is the right answer; they do not, in the legal rhetoric, "substitute their judgment for that of the agency."

Second, as a general rule, the courts do not hear new evidence. When an agency passes a rule, it has before it a body of data, and it makes its decision on the basis of *that* body of data, the "administrative record," as it is called. Decisions are made on a fixed record so that everyone knows what matters were or were not considered. Thus, even if I discover a great theory 4 months after the regulation was passed as to why it is a good regulation, but no one considered that theory when it was written, the court normally will not consider my views. The regulation rises or falls on the evidence in the administrative record, not new evidence added later by agency lawyers or by the courts themselves As the Supreme Court recently said in its *Benzene* decision: "Because our review of this case involved a more detailed examination of the record than is customary, it must be emphasized that we have neither made any factual determinations of our own nor have we rejected any factual findings made by the Secretary." The Supreme Court looked at the preamble, which explains the final rule, and references in that preamble to various scientific data and evaluated OSHA's basis for acting as it did on *that* record.

The rules governing how courts look at rulemaking place great emphasis on the ability to communicate. Science cannot just sit on the shelf. Citizens, lawyers, and judges need to understand each other. When scientists, regulators, and lawyers write a regulation, they must educate themselves so that they can explain the rule to the public and so that it will be clear to a potential reviewing court. Thus, communication skills are very important. Many readers may recall the *Monsanto* case, a challenge to an FDA decision declaring that acrylonitrile, used to make unbreakable beverage containers, was an unsafe food additive. The "legal" issue before the court was the retention of residual acrylonitrile monomer, which had not been polymerized in the manufacturing process. The court decided that it could not be sure whether the Commissioner of Food and Drugs had applied what the court called the second law of thermodynamics, which the court took to define as the diffusion principle, or whether the Commissioner had extrapolated the applicable data. I don't know the difference between projection and extrapolation, and I don't know whether the second law of thermodynamics is synonymous with diffusion or not. (I studied history before I went to law school.) While allegedly asking only whether the agency considered the relevant questions (the standard for the arbitrary and capriciousness test), the court appears to have engaged in considerable scientific evaluation. If the agency carefully states what it is that it has done, if it communicates clearly, then it can encourage courts to limit their review. Because the court found that the Commissioner did not explain which theory he was applying, it was unable to affirm the rule. Thus, communication and drafting skills are part of a court's rulemaking review process, and can affect how much deference a court will give to the agency's scientific decisions.

Let's move from rulemaking to administrative adjudication. What's the legal standard for review for administrative adjudication? The Commissioner must have substantial evidence supporting his position if, for example, he is going to withdraw the New Drug Application. The difference between arbitrary or capricious is becoming difficult to articulate. In a few recent decisions, courts have suggested that the two are more the same than they are different. After all, assuming the agency has considered all the relevant factors, and has a sound basis for making one selection, even though another is conceivable and even defensible, obviously there is some substantial evidence in the agency's favor. Regulatory agencies do not act unless they have a substantial basis to do so.

What "standard" of sound scientific methodology applies to legislative proposals? I think it is fair and adequate to say that it has to be sound enough to withstand the questioning of congressmen and congressional staffers, who, like myself, are likely trained in the social, not physical sciences.

For judicial enforcement the rules are easy,

although lawyers, judges, and scholars have filled rooms with writings on the subject. In an FDA seizure or injunction our science must prevail by a preponderance of the evidence. What's a preponderance? More than 51% and less than 95%. If it's a criminal case, our methodology must be sound beyond a reasonable doubt. That's the 95% degree of confidence area.

There is one simple requirement concerning sound methodology and legal actions when we are talking about enforcement cases. Valid methods win cases; unverified methods don't. In *United States v. Morton-Norwich*, one allegation was adulteration of drugs due to lack of sterility. The court said: "In proving adulteration, the court is not limited to any particular manner of proof. And is especially not limited to use of tests conducted in accordance with the USP. This court was able to review the analytical test sheets and listen to numerous expert witnesses' opinions with regard to the validity of those tests. The court has concluded that those tests conducted were valid and demonstrated that the product was not stable." In a separate but related motion, the defendants moved to exclude certain of the government's sterility test results because FDA did not "preserve and produce records relating to the preparation and sterilization, by autoclaving, of the culture medium used in the testing." The Court denied the motion: "In light of the use by the FDA analysts of both positive and negative controls, all of which included results indicative of satisfactory testing conditions, the court determines that the results of the tests are accurate and relevant and, therefore, should not be excluded." There are not as many court opinions in FDA enforcement cases as might be expected because, much like other law enforcement cases, the great majority of them are settled by a plea or consent. There is no trial. Thus, not every example of sound methodology is recorded.

What is the message? Courts are telling us that if we want to rely on something scientific, we must prove it. After we have proved it, we must explain it. And when it comes to that form of legal action called rulemaking, we must explain it in a certain way—on the record, at one point in time. We can't pass a rule today and develop the requisite data to substantiate it 6 months later. Therefore, regulatory scientists must continue to emphasize method validation, continue to use the appropriate controls, whatever they may be, and fulfill their obligation as scientists working for regulatory agencies to be able to explain their actions. Those communications skills may be just as important as the science that was done; not to scientific colleagues, who are capable of understanding scientific data, but to people like me who work with the regulatory scientist and to other citizens we ultimately work for.

INDUSTRIAL CHEMICALS

Glass Capillary Gas Chromatography for Sensitive, Accurate Polychlorinated Biphenyl Analysis

BRIAN BUSH, STEVEN CONNOR, and JOHN SNOW
New York State Department of Health, Department of Laboratories, Albany, NY 12201

A simple method is described for preparing Apiezon L-coated glass capillary columns for polychlorinated biphenyl (PCB) analysis. The mass represented by each of 72 chromatographic peaks separated on this column from a mixture of Aroclors 1221, 1016, 1254, and 1260 was determined; the mixture could then be used as a universally applicable calibration standard for analysis of environmentally modified polychlorinated biphenyl mixtures at the parts per billion (ng/g) level. Analytical sensitivity is <10 ng/mL for total PCBs and <0.1 ng/mL for individual PCB congeners.

Quantitative analysis of polychlorinated biphenyls (PCBs) in the environment has been performed, to date, almost exclusively on packed gas chromatographic columns with electron capture or Hall electrolytic conductivity detection. Because of the inability of the columns to separate PCB mixtures into their individual components, quantitation methods have been empirical (1). Earlier methods in which patterns for PCB residues did not closely match commercial PCB-mixture patterns were obviously unsatisfactory. A peak-by-peak method proposed by Albro and Fishbein in 1972 (2) and Webb and McCall in 1973 (3) was shown in a collaborative study (4) to be superior to earlier, more empirical methods and was adopted by AOAC as official first action (5). However, the present *Federal Register* method (6) has reverted to an entirely empirical procedure.

The great majority of PCB congeners could be separated on SCOT capillary columns (7), but analysis times were long and modern microliter injection systems were not then available, precluding routine trace analysis. As a result of considerable developmental innovation during the past 10 years (8), glass capillary columns are manufactured simply and inexpensively, so true compound-by-compound analysis is possible (9). Retention times have been reduced by using thin, wall-coated films and temperature programming, and the resultant sharp peaks allow sensitive quantitation.

Received July 13, 1981. Accepted November 4, 1981.

This paper describes a rapid, accurate analysis for PCB congeners based on glass capillary technology with Apiezon L as the stationary phase. Apiezon L is the most selective phase for PCB congeners (10, 11) and has the additional advantage that peaks have been assigned structures by Sissons and Welti (10, 12) and by Jensen and Sundström (13) on a closely related packed column.

The group of compounds addressed are the chlorinated hydrocarbons separated by a simple Florisil-based cleanup method (14) and found in the northeast United States: PCB mixtures Aroclors 1221, 1242, 1248, 1254, and 1260 (manufactured by Monsanto Co.), plus hexachlorobenzene (HCB), octachlorostyrene, mirex, and *p,p'*-DDE. The same method can be used to quantitate *o,p'*-DDE and photo-mirex, but because these are rarely found in the environment they were not included in the detailed study.

Seventy-eight chromatographic peaks were measured in 30 min or less, and a PDP 11/45 computer was used to calculate the concentrations.

Experimental

Apparatus and Reagents

(a) *Gas chromatograph.*—Hewlett-Packard 5840A with Hewlett-Packard 5880 capillary injection system used in the splitless mode, autosampler with 2 μL injection volume, electron capture detector, extended memory, and ASCII computer interface boards. Typical operating conditions: column-head pressure 15–20 psi helium; initial temperature 70°C; initial time 0 min; program rate 10°/min to 130°C, 4°/min to 230°C, and hold for 10 min. Columns differ slightly; vary conditions as needed to separate mirex, HCB, and *p,p'*-DDE from all PCBs. This normally results in linear gas velocity of 25–30 cm/s. Switch valve to purge at 0.5 min after injection. Other conditions: argon–methane (95 + 5) make-up gas flow 30 mL/min; injection 250°C; electron capture detector 300°C; recorder chart 1 cm/min.

0004-5756/82/6503-0555–12$01.00

Table 1. Comparison of chromatographic peaks

Retention time, min	Assigned structure	Sissons & Welti No.	Jenson & Sundstrom No.	Authentic sample supplier[a]	No. of Cl atoms by MS
5.33	2	2	—	A	1
6.59	2,2′	3	—	A	2
6.79	4	4	—	A	1
7.86	2,5′	5	—	A	2
7.98	2,4	6	—	A	2
8.10	2,3′	7	—	A	2
8.21	2,3	—	—	A	2
8.36	2,4′	8	—	A	2
9.37	2,2′5′	9	—	A	3
9.62	2,2′4′	10	—	A	3
9.79	2,2′3′ + 3,2′6′	11	—	—	3
10.18	4,2′6′	12	—	—	3
10.41	4,4′	13	—	A	2
11.09	2,2′4′6′	14	—	—	4
11.32	3,2′5′	15	—	A	3
11.39	2,3′5′	16	—	—	3
11.49	Cl3	—	—	—	3
11.55	Cl3	—	—	—	3
11.65	3,2′4′	17	—	R	3
11.85	3,2′3′ + 4,2′4′	18,19	—	A	3
12.12	2,3′4′	20	—	—	3
12.62	25,2′5′	21	1	A	4
12.90	24,2′5′	22	2	A	4
13.07	23,2′5′	24	3	A	4
13.22	24,2′4′	—	—	A	4
13.40	Cl4	—	—	—	4
13.56	23,2′,3′	27	—	—	4
13.65	Cl4	—	—	—	4
13.82	Cl4	—	—	—	4
14.52	Cl5	—	—	—	5
	25,2′3′6′	29	6	R	5
15.03	23,2′3′6′	32	—	—	5
15.45	25,3′4′	33	—	A	4
15.66	24,3′4′	35	—	A	4
15.96	25,2′3′5′	—	10	—	—
16.03	4,2′3′4′	36	—	—	5
16.13	236,2′3′6′	—	11	A	—
16.36	25,2′4′5′	39	12	A	5

(b) *Apiezon L.*—R.F.R. Corp., Hope, RI 02831.

(c) *Glass-drawing machine.*—GDM-1, Shimadzu Scientific Instruments, Inc., Columbia, MD. Oven temperature 760°C; bent-pipe temperature setting 64 for Pyrex glass tubing (7 mm od, 3 mm id); gear ratio 80 for 0.34–0.35 mm id capillary ca 60 m long, or 100 for 0.29–0.31 mm id capillary ca 80 m long.

(d) *Microcolumn treating stand.*—MCT 1A, Shimadzu Inc. Used only to provide easy connection to variable nitrogen pressure source.

(e) *PCB standards.*—Commercial mixtures were provided by Monsanto Co. in 1971, except for Aroclor 1016, which was provided in 1973. No lot numbers were given. Individual PCB congeners were obtained from suppliers shown in Table 1. Although some samples contained detectable impurities when examined by the gas chromatographic (GC) system described here, no impurity contributed more than 1% to total electron capture signal produced by the compound.

(f) *Pesticide standards.*—Obtained from Environmental Protection Agency Pesticide Repository, Research Triangle Park, NC.

(g) *Solvents.*—Omnisolve grade (MCB, Inc., Cincinnati, OH 45212).

(h) *Mercury.*—Redistilled (Fisher Scientific Co., Fair Lawn, NJ).

(i) *Gases.*—Ultra-high purity for GC; oxygen traps maintained for helium carrier gas; hydrogen chloride (Matheson, East Rutherford, NJ).

Capillary Column Preparation

Wash glass tubing successively with acetone, dichloromethane, aqueous KOH (1.0%), and methanol before drawing into capillary. De-

Table 1. (continued)

Retention time, min	Assigned structure	Sissons & We ti No.	Jenson & Sundstrom No.	Authentic sample supplier[a]	No. of Cl atoms by MS
16.68	24,2'4'5'	41	13	—	5
16.85	23,2'4'5'	42	14	—	5
16.92	25,2'3'4'	43	15	—	5
17.37	23,2'3'4'	44	16	—	5
17.66	25,2'3'5'6'	—	20	—	6
17.97	23,2'3'5'6'	—	21	—	6
18.16	234,2'3'6'	47	22	—	6
18.50	Cl5	—	—	—	5
18.78	236,2'3'5'6'	51	24	—	7
18.95	34,2'4'5'	52	25	—	5
19.28	236,2'3'4'6'	—	26	—	7
19.44	235,2'4'5'	—	27	—	6
19.72	Cl5	—	—	—	5
19.87	34,2'3'4'	52	28	—	5
20.06	245,2'4'5'	56	29	—	6
20.29	Cl6	—	—	—	6
20.46	Cl6	—	—	—	6
20.66	234,2'4'5'	59	32	—	6
20.87	235,2'3'5'6'	—	33	—	7
21.01	245,2'3'5'6'	—	36	—	7
21.18	Cl7	—	—	—	7
21.27	234,2'3'4'	52	37	—	6
21.51	2356,2'3'5'6'	—	41	—	8
21.79	234,2'3'4'6'	55	42	—	7
22.13	245,3'4'5'	56	44	—	6
22.58	236,2'3'4'5'6'	—	45	—	8
23.13	34,2'3'4'5'	54	—	—	6
23.27	235,2'3'4'5'	—	48	—	7
23.63	245,2'3'4'5'	—	49	—	7
24.22	234,2'3'4'5'	—	50	—	7
24.37	2345,2'3'5'6'	—	51	—	8
24.56	Cl7	—	—	—	7
24.71	2345,2'3'4'6'	—	53	—	8
24.86	245,2'3'4'5'6'	—	54	—	8
25.49	234,2'3'4'5'6'	—	56	—	8
27.27	345,2'3'4'5'	—	58	—	ND[b]

[a] A = Analabs Inc.; R = RFR Corp.
[b] Not detected.

termine capillary dimensions by weighing one turn before and after filling with water. Straighten ends of remaining column, fill column with HCl gas at 5 psi, and seal ends. Place column in muffle furnace 3 h at 350°C. Remove from furnace and cool. Blow out HCl gas with nitrogen. Draw in hexamethyl disilazane to fill half column length, and then expel it under nitrogen pressure (10 psi). Rinse column with one volume of toluene under suction, and dry 2 h with nitrogen.

Draw isooctane solution of Apiezon L (2%) into column under suction until column is full; then draw in plug of mercury, ca 4 cm long. Allow air space to form, and then introduce second plug of mercury. Force coating solution out of column into secondary capillary ca 10 m long under nitrogen pressure sufficient to maintain coating speed of 2 cm/s. Second column is attached to prevent coating solution from accelerating when end of column is approached. Maintain nitrogen flow through column for 2 h to evaporate residual isooctane. Install column in the GC apparatus, and condition at 0.5°C/min to 270°C. Hold overnight

Method Calibration

Peak structure assignment.—Figure 1 shows the separation of 72 PCBs (a mixture of Aroclors 1221, 1016, 1254, and 1260) on a typical column (20 m × 0.29 mm). Aroclors 1242 and 1248 contain 3,4,3',4'-tetrachlorobiphenyl in small proportion. This congener is important toxicologically (15) and is also available commercially; therefore, we add it to the calibration mixture of Aroclors so that it may be quantitated, and also used to in-

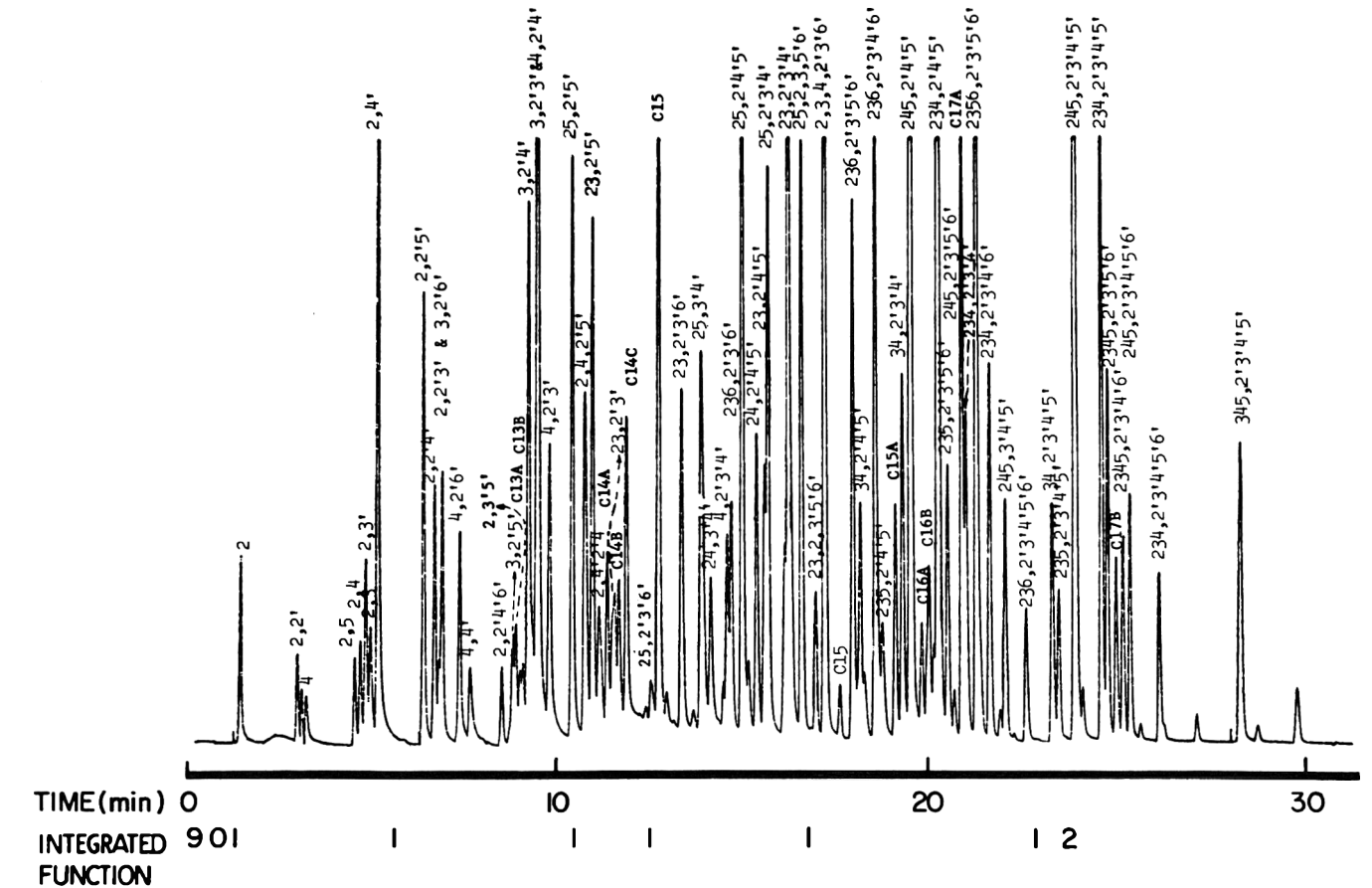


Figure 1. Chromatogram of mixture of Aroclors 1221, 1016, 1254, and 1260 (2 μg/mL each) on 29 m glass capillary column. Integrator functions are shown; conditions are given in text.

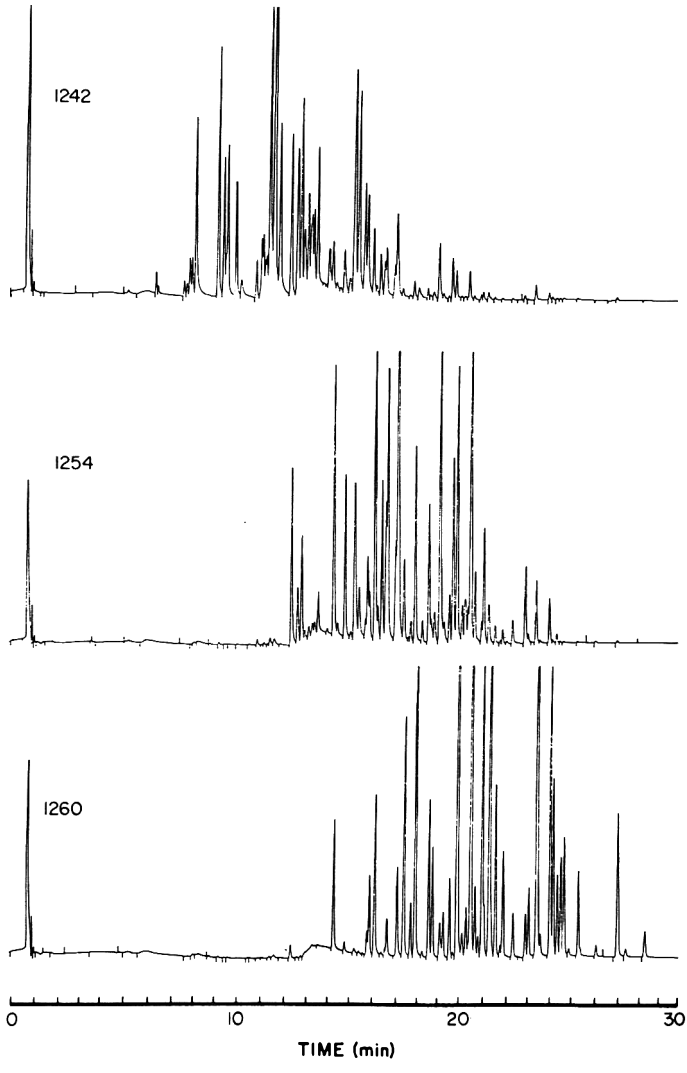


Figure 2. Chromatograms of Aroclors 1242, 1254, and 1260.

dicate the presence of residues of these 2 Aroclor mixtures. Figure 2 shows the chromatograms of individual Aroclor mixtures. We assigned structures by comparing both the retention times and pattern of peaks with the Sissons and Welti (10) chromatograms of Aroclors 1242, 1254, and 1260. We also assigned structures (Table 1) by comparing retention times with those in another study with Apiezon L, by Jensen and Sundström (13), and with those of authentic samples of synthetic PCB. In addition we determined the chlorine content of each peak by mass spectrometry using a resolving power of 5000–7000 (Kratos MS-25 with Carlo Erba chromatograph and jet molecular separator).

Where the previous authors were undecided, we have simply named the peak Cl_n where n is the number of chlorines in the molecule. When there are several isomers they are also given a letter, e.g., Cl_5A, Cl_5B, etc., to make computer treatment of the data possible.

Integrator operation.—Of the options available with the Hewlett-Packard 5840 chromatograph integrator, those used (Figure 1) were applied at the time appropriate for each column. Their locations on the standard PCB chromatogram are always as shown. The setting "9" indicates "Inhibit bunching," which also renders inoperative a particular Hewlett-Packard routine which is unsuitable for this work. The setting "0" in-

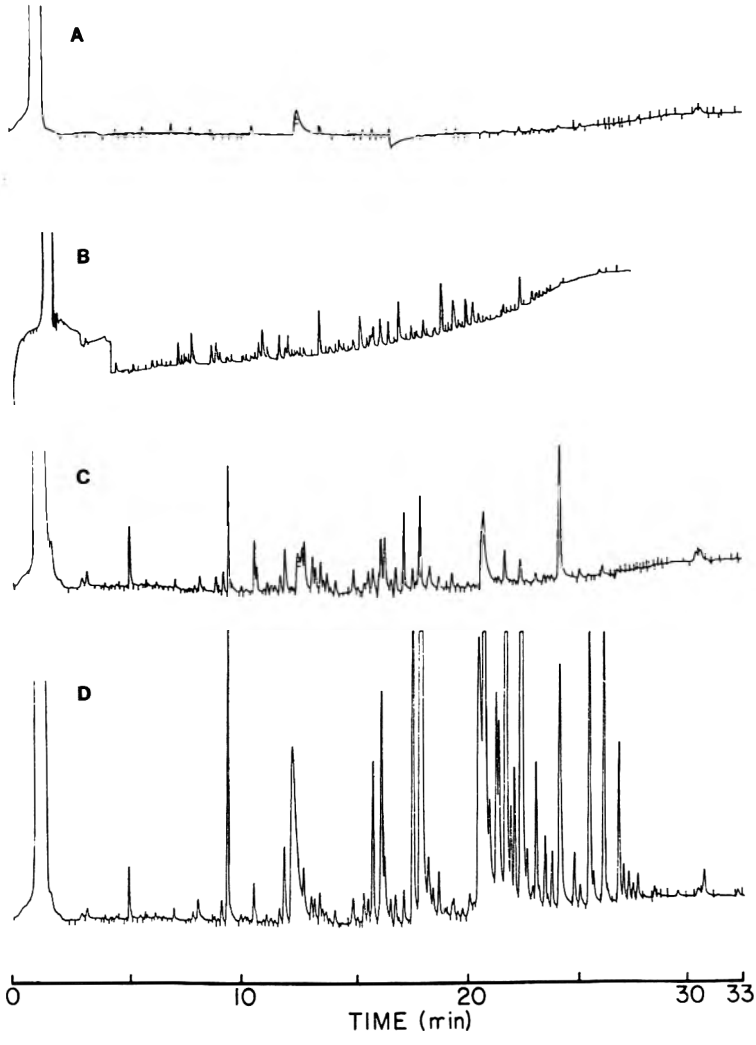


Figure 3. Chromatograms of (A), solvent blank; (B), 10 ng/mL of a mixture of Aroclors 1221, 1016, 1254, and 1260; (C), bovine milk sample concentrated 10 times; (D), human milk sample concentrated 15.6 times.

dicates "Set baseline now." The setting "1" indicates "Set baseline at next valley"—a function well suited to the complex chromatogram of PCB. The setting "2" indicates "Reset baseline at all valleys." At the point shown, it compensates for the sloping baseline, which is not apparent in the Figure 1 chromatogram of a 2 μg/mL solution, but is apparent when more dilute solutions (10–100 ng/mL) are run (Figure 3).

Determination of response coefficients.—Figure 4 shows the chromatogram of a mixture of 29 PCBs (Table 1) dissolved at a concentration of 1 ng/mL isooctane. The second chromatogram of the same mixture is reproduced to illustrate the great improvement in efficiency achieved with the glass capillary column. A similar comparison can be made by comparing Figure 1 with the chromatogram in the report by Pastel et al. (14) which specified the same packed column shown in Figure 4.

Response coefficients for each PCB were calculated and plotted against chlorine number (Figure 5). A quadratic curve was fitted to the points by using a least squares fitting routine.

The complete mixture of commercial products (Figure 1) was then analyzed; response coefficients derived from the regression curve were used for peaks for which no standard sample was

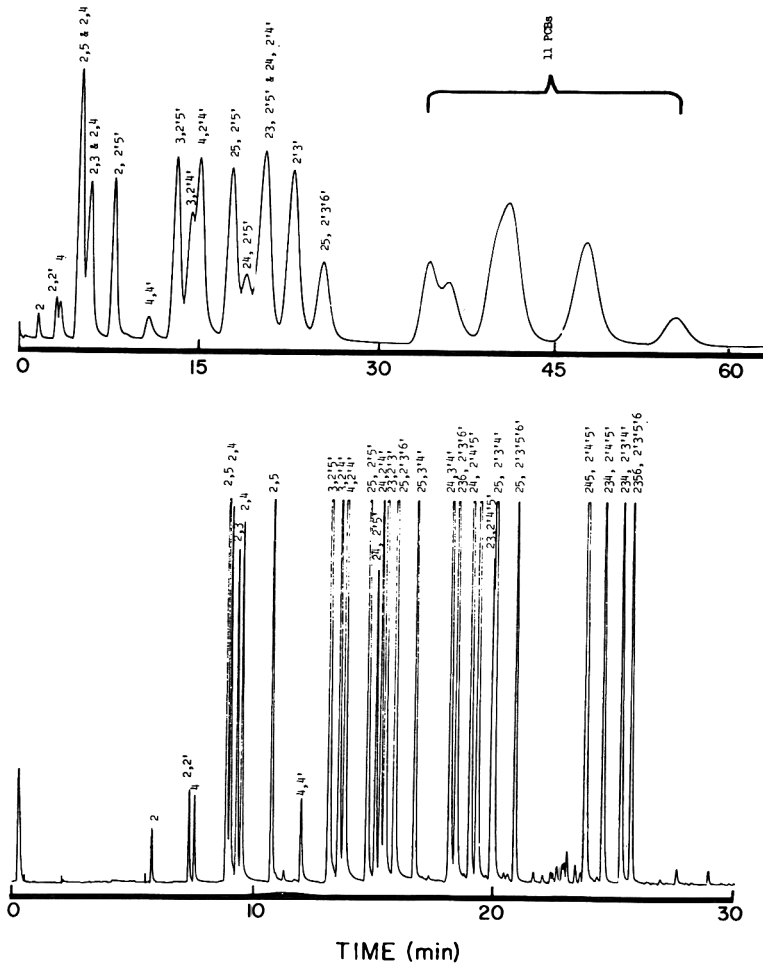


Figure 4. Chromatogram of the same mixture of 0.1 μg/mL each of 29 authentic PCB congeners: (top), on packed Apiezon L column (conditions in Ref. 13); and (bottom), on glass capillary column.

available. The results are given in Table 2, column 3, along with the quantities found in the individual commercial products (columns 4–7) and the sum of those quantities (last column).

Data Handling

The GC reports are transmitted to a cassette deck (Silent 700) via the ASCII interface and processed batchwise with the PDP 11/45 computer program which applies the dilution factor to the result for each congener and pesticide and lists the results with the congener's structure. It also sums the PCB concentrations, reports "Total PCBs," and (where appropriate) prints sample identification data.

Results and Discussion

Peak Assignments

PCB structures shown in Figure 1 and in the subsequent tables were deduced by 4 different procedures. The first step relies heavily on the work of Sissons and Welti (10). Their work with an Apiezon L SCOT column is particularly reliable because it combined mass spectrometry and nuclear magnetic resonance at 220 MHz with an innovative use of retention index (½ RI values) to predict substitution patterns. The latter is a development of the original theory of partition chromatography of Martin and Synge (16), which was exploited by I. E. Bush in steroid structure determination by paper partition

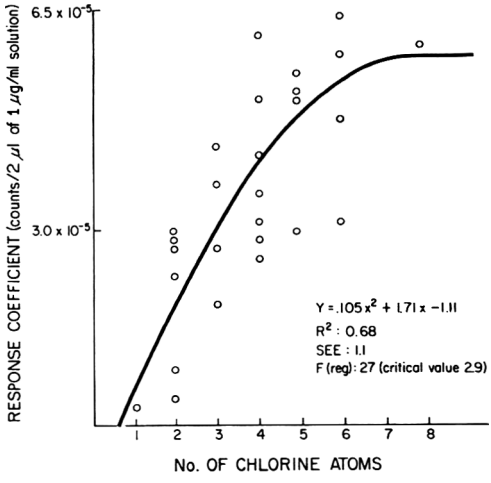


Figure 5. Response coefficient as function of number of chlorine atoms per molecule for 29 authentic PCBs which occur in the environment (identified in Figure 3, bottom, and Table 1).

chromatography (17). We have related our chromatograms to those of Sissons and Welti first by pattern matching and then by measuring retention times relative to a prominent peak in each Aroclor mixture (Cl_3A in Aroclor 1242; 2,4,5,2′,4′,5′-hexachlorobiphenyl in Aroclor 1254; 2,4,5,2′,3′,4′,5′-heptachlorobiphenyl in Aroclor 1260) by using isothermal chromatography. This process was started with packed columns in 1973 (18). The procedure was also applied to Jensen and Sundström's work (13); they provide additional structural data indicating *o*-chlorination by preliminary separation on carbon.

Once peaks had been assigned structures, temperature programming was commenced for analytical convenience. Analysis times were drastically reduced and peaks were sharpened, improving sensitivity of integration. The overall pattern of peaks did not change with programming. We have never observed a change in elution order of the PCBs at different temperatures. It is possible that the order changes reported by Sissons and Welti could have been the result of an adsorptive interaction with the support material of the SCOT tube. Molecules with a different carbon skeleton do behave differently as predicted and observed for steroid skeletons by I. E. Bush. This effect is used in the present work for resolving mirex, HCB, and *p,p′*-DDE.

The primary peak assignments were next checked by comparing retention times of 29 commercially available authenticated standard materials. Finally, the chlorine substitution numbers of the peaks in the programmed run were determined, using a resolving power of 5000–7000.

Because peak structures were deduced by empirical means, the confidence in each assignment is different. It will be some time before enough data are accumulated to assign structures to all observed peaks by the stringent standards normally laid down for synthetic chemical authentication. However, the availability of synthesized and characterized compounds is increasing (19) and, although chromatographic matching of retention parameters does not necessarily constitute perfect authentication of a peak assignment, several authors are reaching a consensus on the structure of compounds separated on Apiezon L (19, 20) and other phases (9). We consider that the structures of the major peaks are now well enough established to warrant reporting them quantitatively as described here. This will enable the task of determining the toxicological significance of residues of PCB to be started, a hopeless task when results are expressed in terms of Aroclor mixtures as has been the practice hitherto.

Accuracy

The accuracy of the method can be assessed by examining the totals in Table 2. Only 29 of the 72 values are based on true response coefficients. Despite this, the totals for each component and for each complete mixture are close to the expected totals, i.e., 2.0 μg/mL for individual Aroclors and 8 μg/mL for all four. There is also agreement between the calibrated amount in a complete mixture and the sum of Aroclors determined separately. Although repeating the process one or more times and adjusting the estimated response coefficients slightly at each iteration would have produced greater exactitude, this was considered unnecessary in view of the accuracy normally accepted in PCB analysis.

The total for Aroclor 1221 is obviously too high in view of the fact that this mixture contains unchlorinated biphenyl. However, this Aroclor mixture rarely occurs in the environment alone and, when it occurs in a mixture, the only peak unique to it (by which it can be estimated) is 2-chlorobiphenyl. More effort to obtain an accurate sum is thus unwarranted. Also the trace contributions of peaks later than 2,4′-dichlorobiphenyl are ignored for the summation.

Precision

Because so many concentrations are summed, it is possible that errors in the individual concentrations would cancel one another out. Replicate analyses were therefore carried out with a mixture of synthetic PCBs. Table 3 shows the results for 6 repetitive runs. Theoretically, the error in the total, obtained by summing the 27 results, should equal the square root of the sum of the squares of the absolute standard deviations (21), or ±0.06. The observed standard deviation of 6 sums is ±0.125—surprisingly good agreement, considering the complexity of the task the integrator performs.

Clearly, the less well separated peaks are integrated less precisely than the well separated peaks. For 2-dichlorobiphenyl, the integrator precision is at its worst, both because of baseline irregularities resulting from injection and because of the very rapid changes in signal this early in this chromatogram.

Day-to-day reproducibility is illustrated in Table 4.

Linearity

The response of most electron capture detectors is not linear with concentration. In this work, the response for 15 PCBs from monochlorinated biphenyls through hexachlorinated biphenyls was observed from 0 to 4 μg/mL. All showed a common quasi-linear curve from 0 to 1 μg/mL and a second quasi-linear portion from 1 to 4 μg/mL. In the mixed solutions shown in Table 2, all responses lie within the linear range, even for 2-chlorobiphenyl, which has a particularly low response coefficient (Figure 5). Clearly all samples should be diluted, if necessary, so that

Table 2. Analysis of 1:1:1:1 mixture and separate Aroclor mixture

Retention time, min.	Structure	Calib. amt, μg/mL	Aroclor (2.0 μg/mL) 1221	1016	1254	1260	Sum of Aroclors
5.33	2	0.794	1.264				1.264
6.59	2,2′	0.182	0.118	0.094			0.212
6.79	4	0.425	0.400				0.400
7.86	2,5	0.026	0.018	0.008			0.026
7.98	2,4	0.035	0.033	0.008			0.041
8.10	2,3′	0.092	0.069	0.034			0.103
8.21	2,3	0.047	0.030	0.026			0.059
8.36	2,4′	0.323	0.213	0.166			0.379
9.37	2,2′5′	0.216	T[a]	0.185			0.202
9.62	2,2′4′	0.074	T	0.065			0.073
9.79	2,2′3′ + 3,2′6′	0.096	T	0.094			0.102
10.18	4,2′6′	0.074	T	0.057			0.074
10.41	4,4	0.158	0.104	0.064			0.168
11.09	2,2′4′6′	0.018	T	0.015			0.018
11.32	3,2′5′	0.040	T	0.032			0.039
11.39	2,3′5′	0.038	T	0.034			0.037
11.49	Cl3	0.021	T	0.017			0.020
11.55	Cl3	0.025	T	0.023			0.024
11.65	3,2′4′	0.167	T	0.149			0.166
11.85	3,2′3′ + 4,2′4′	0.267	T	0.238			0.271
12.12	4,2′3′	0.140	T	0.127			0.147
12.62	25,2′5′	0.233	T	0.115	0.133		0.268
12.90	24,2′5′	0.151	T	0.113	0.042		0.161
13.07	23,2′5′	0.166	T	0.105	0.067		0.179
13.22	24,2′4′	0.057	T	0.047	0.011		0.063
13.40	Cl4	0.050		0.039	0.008		0.047
13.56	23,2′3′	0.049		0.039	0.011		0.050
13.65	Cl4	0.050		0.043	0.015		0.058
13.82	Cl4	0.110		0.107	0.057		0.164
14.52	Cl5	0.165			0.115	0.081	0.196
15.03	23,2′3′6′	0.085			0.078	0.025	0.103
15.45	25,3′4′	0.134			0.105	0.013	0.118
15.66	24,3′4′	0.042			0.032	0.006	0.038
16.03	4,2′3′4′	0.053			0.059	0.015	0.074
16.13	236,2′3′6′	0.086			0.041	0.056	0.097
16.36	25,2′4′5′	0.181			0.136	0.072	0.208
16.68	24,2′4′5′	0.070			0.069	0.006	0.075
16.85	23,2′4′5′	0.080				Not integrated	

Table 2. (continued)

Retention time, min.	Structure	Calib. amt, μg/mL	Aroclor (2.0 μg/mL) 1221	1016	1254	1260	Sum of Aroclors
16.92	25,2'3'4'	0.116			0.149	0.024	0.173
17.37	23,2'3'4'	0.269			0.289	0.049	0.338
17.66	25,2'3'5'6'	0.112			0.037	0.087	0.124
17.97	23,2'3'5'6'	0.029			0.012	0.021	0.033
18.16	234,2'3'6'	0.206			0.083	0.145	0.228
18.50	Cl5	0.014			0.013	0.007	0.020
18.78	236,2'3'5'6'	0.096			0.051	0.054	0.105
18.95	34,2'4'5'	0.058			0.015	0.051	0.066
19.28	236,2'3'4'6'	0.135			0.128	0.017	0.145
19.44	235,2'4'5'	0.032			0.015	0.020	0.035
19.72	Cl5	0.051			0.024	0.034	0.058
19.87	34,2'3'4'	0.082			0.080	0.005	0.085
20.06	245,2'4'5'	0.369			0.127	0.253	0.380
20.29	Cl6	0.025			0.015	0.011	0.026
20.46	Cl6	0.045			0.027	0.021	0.048
20.66	234,2'4'5'	0.266			0.130	0.165	0.295
20.87	235,2'3'5'6'	0.051			0.035	0.028	0.063
21.01	245,2'3'5'6'	0.010				0.008	0.008
21.18	Cl7	0.118			0.009	0.127	0.136
21.27	234,2'3'4'	0.053			0.040		0.040
21.51	2356,2'3'5'6'	0.193			0.022	0.170	0.192
21.79	234,2'3'4'6'	0.068			0.011	0.060	0.071
22.13	245,3'4'5'	0.048			0.008	0.047	0.055
22.58	236,2'3'4'5'6'	0.026			0.009	0.018	0.027
23.13	34,2'3'4'5'	0.047			0.033	0.019	0.052
23.27	235,2'3'4'5'	0.029			0.005	0.023	0.028
23.63	245,2'3'4'5'	0.278			0.027	0.250	0.277
24.22	234,2'3'4'5'	0.120			0.020	0.102	0.122
24.37	2345,2'3'5'6'	0.064			T	0.059	0.064
24.56	Cl7	0.033			T	0.028	0.035
24.71	2345,2'3'4'6'	0.035			T	0.032	0.036
24.86	245,2'3'4'5'6'	0.042			T	0.038	0.043
25.49	234,2'3'4'5'6'	0.033				0.030	0.030
27.27	345,2'3'4'5'	0.065			T	0.062	0.063
	Total	8.238	2.249	2.044	2.393	2.339	9.225

[a] T = Trace.

Table 3. Integrator precision for 6 replicates

Structure	Mean, μg/mL	SD	RSD, %	Structure	Mean, μg/mL	SD	RSD, %
2	0.375	0.031	8.26	24,2'4'	0.273	0.008	2.93
2,2'	0.215	0.005	2.33	23,2'3'	0.306	0.006	1.96
4	0.484	0.021	4.34	25,3'4'	0.200	0.003	1.50
2,5	0.140	0.006	4.28	24,3'4'	0.122	0.002	1.64
2,4	0.104	0.003	2.88	236,2'3'6'	0.279	0.004	1.43
2,3	0.085	0.002	2.35	25,2'4'5'	0.234	0.019	8.12
2,4'	0.175	0.006	3.43	23,2'4'5'	0.285	0.017	5.96
2,2'5'	0.253	0.008	3.16	25,2'3'5'6'	0.085	0.001	1.17
4,4'	0.149	0.008	5.37	245,2'4'5'	0.282	0.007	2.48
3,2',5'	0.316	0.009	2.85	234,2'4'5'	0.087	0.002	2.30
3,2'4'	0.148	0.004	2.70	234,2'3'4'	0.081	0.002	2.47
4,2'4'	0.228	0.007	3.07	2356,2'3'5'6'	0.090	0.005	5.55
25,2'5'	0.319	0.007	2.19				
24,2'5'	0.119	0.003	2.52				
23,2'5'	0.117	0.003	2.56				
				Total[a]	5.5	0.125	2.25

[a] Known 5.47 μg/mL.

Table 4. Day-to-day reproducibility of total PCBs

Mixtures, (μg/mL)	Calibr. amt. μg/mL	Day 1, μg/mL			Day 4, μg/mL			Mean, μg/mL	SD	RSD, %
All 29 (5.8)	5.8	5.473	5.441	5.676	5.423	5.605	5.709	5.554	0.125	2.3
1221 (2.0)	2.0	2.145			1.99					
1016 (2.0)	2.0	2.083			1.919					
1254 (2.0)	2.0	2.392			2.548					
1260 (2.0)	2.0	2.339			2.494					
Sum (8.0)	8.2	8.475	8.296	7.784	8.601	8.048		8.241	0.329	4.0

the concentration of each individual component is <1 μg/mL.

Detection Limit

Defining the detection limit for this type of analysis is not easy; it is probably not so much a function of the GC instrumentation alone as of the total analytical system. For example, as the signal from the sample decreases, the integrator fails to detect larger and larger peaks. Also small peaks tend to be incorporated into larger peaks. Often analytical sensitivity is determined in practice by the residual impurities in the solvents which was the case with Tessari and Savage (22) whose practical detection limit was approximately 50 ng/mL for Aroclor 1254. Figure 3 shows the chromatogram of a solution containing 10 ng/mL of each of the 4 Aroclors treated here, along with chromatograms of a solvent blank and 2 samples of milk. The overall detection limit lies in the region from 1 to 5 ng/g for the milk but is as low as 0.05 ng/g for indi-

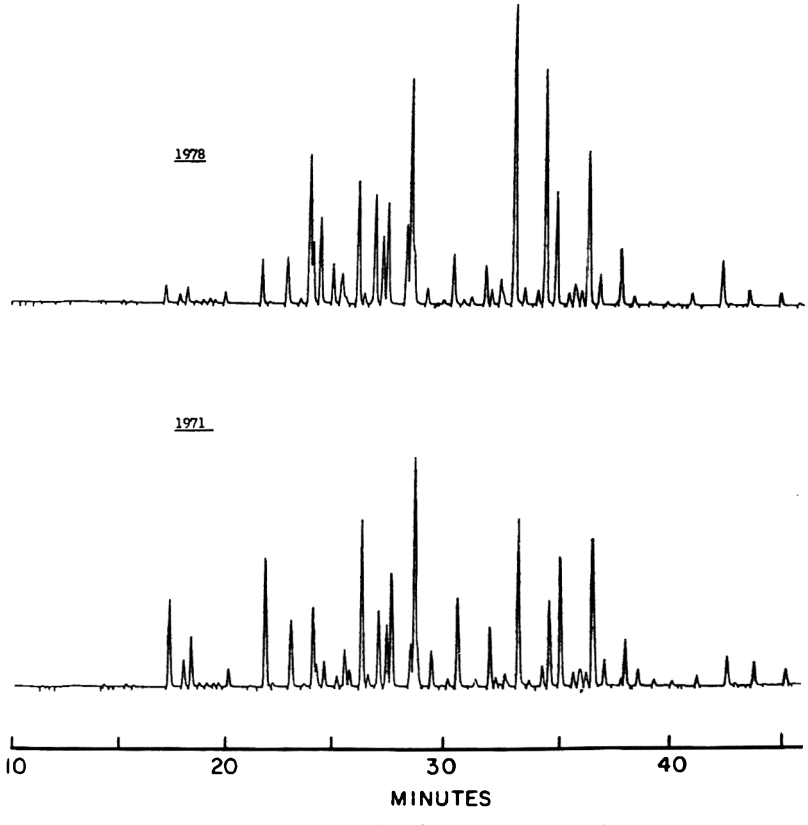


Figure 6. Chromatograms of Aroclor 1254 (2 μg/mL hexane) obtained from Monsanto, Inc. in 1971 and 1978.

vidual components. This high sensitivity is due in large part to the rapid concentration changes produced by the efficient chromatography, allowing very small peaks to be detected.

Application

The method can be applied to purified PCB residues from any matrix. Calibration can be achieved as described here. Once the Aroclor mixtures available for calibration have been analyzed, the mass represented by each peak is applicable until that batch of standard material is exhausted, regardless of the chromatographic equipment used. If resolution of various peaks becomes impossible or if time is not available to achieve it, the masses can be combined and the peaks reported as mixtures, for a first approximation.

The method is also useful for quality assurance for laboratories using packed-column technology. This laboratory was recently called on to explain systematic errors found between laboratories participating in an assurance program. Figure 6 illustrates the cause of this error quite clearly: The manufacturer changed from a batch to a continuous process during the 1970s (J. A. Liddle; Private Communication) and a different pattern of PCB congeners resulted.

The method can also be employed for chromatographic fractions containing a limited number of other chlorinated xenobiotics commonly found in the biological matrices for which this method is primarily designed. In our region, after the simple one-step Florisil cleanup (14), those commonly found are HCB, *p,p'*-DDE, octachlorostyrene (in Lake Ontario fish samples), and mirex.

REFERENCES

(1) Bush, B., Baker, F., Dell'Acqua, R., Houck, C., & Lo, F. C. (1975) *J. Chromatogr.* **109,** 287–295
(2) Albro, P. W., & Fishbein, L. (1972) *J. Chromatogr.* **69,** 275–283
(3) Webb, R. G., & McCall, A. C. (1973) *J. Chromatogr. Sci.* **11,** 366–372
(4) Sawyer, L. D. (1973) *J. Assoc. Off. Anal. Chem.* **56,** 1015–1023
(5) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Arlington, VA, Sec. **29.018**
(6) *Fed. Regist.* (1979) **44**(233), 69501–69509
(7) Sissons, D., & Welti, D. (1971) *J. Chromatogr.* **60,** 15–32
(8) Lee, M. L., & Wright, G. W. (1980) *J. Chromatogr.* **184,** 235–312
(9) Albro, P. W., Corbett, J. T., & Schroedar, J. L. (1981) *J. Chromatogr.* **205,** 103–111
(10) Sissons, D., & Welti, D. (1971) *J. Chromatogr.* **60,** 15–32
(11) Albro, P. W., Hoseman, J. K., Clemmer, T. A., & Corbett, B. J. (1977) *J. Chromatogr.* **136,** 147–153
(12) Welti, D., & Sissons, D. (1972) *Org. Magn. Reson.* **4,** 309–319
(13) Jensen, S., & Sundström, G. (1974) *Ambio* **3,** 70–75
(14) Pastel, M., Bush, B., & Kim, J. S. (1980) *Pestic. Monit. J.* **14,** 11–12
(15) Yoshimura, H., Yoshihara, S., Ozawa, N., & Miki, M. (1979) *Ann. N. Y. Acad. Sci.* **320,** 179–192
(16) Martin, A. J. P., & Synge, R. L. M. (1941) *Biochem. J.* **35,** 1358–1372
(17) Bush, I. E. (1961) *The Chromatography of Steroids,* Pergamon Press, New York, NY
(18) Bush, B., Tumasonis, C. R., & Baker, F. D. (1974) *Arch. Environ. Contam. Toxicol.* **2,** 195–212
(19) Mullin, M., Sawka, G., Safe, L., McCrindle, S., & Safe, S. (1981) *J. Anal. Toxicol.* **5,** 138–142
(20) Ballschmiter, K., & Zell, M. (1980) *Fresenius Z. Anal. Chem.* **302,** 20–31
(21) *Experimental Statistics* (1966) Handbook 91, U.S. Departmen: of Commerce, Washington, DC
(22) Tessari, J. D., & Savage, E. P. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 736–741

FLAVORS AND NONALCOHOLIC BEVERAGES

Rapid High Performance Liquid Chromatographic Determination of Monosodium Glutamate in Food

PETER SPORNS
The University of Alberta, Department of Food Science, Edmonton, Alberta, Canada T6G 2P5

A rapid, accurate high performance liquid chromatographic method is described for determination of glutamic acid in food. Average recovery of added glutamic acid was 99.2% by this method. The method could be used to analyze samples such as soy sauce, which contain a large amount of other potentially interfering soluble compounds.

Monosodium glutamate (MSG) is an important food additive. In the United States alone, in 1979, the market for MSG was valued at about fifty-five million dollars (1).

Both consumers and food processors have an interest in monitoring levels of MSG in food. The main consumer concern is a condition which has come to be known as "Chinese Restaurant Syndrome" (2). The U.S. Food and Drug Administration Select Committee on GRAS Substances reported "there is no evidence in the available information on . . . (all common forms of glutamate) . . . that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when they are used at levels that are now current and in the manner now practiced." But in the same report they go on to state that some people will react to relatively small doses of MSG and that further research to evaluate this reaction is under way (3). The food processor is interested in adding MSG to food at levels which provide the best flavor enhancement at the lowest cost, usually levels from 0.2 to 0.8% MSG (3).

Various methods for detecting MSG can be found in the literature. A paper chromatographic method (4) has been reported, which requires overnight development of paper chromatograms after extraction of MSG, followed by an approximate estimation of MSG concentrations by comparison with similarly chromatographed standards. The AOAC official method (5), originally reported by Fernandez-Flores et al. (6), involves extraction of MSG followed by a time-consuming ion exchange separation and Sorenson formol titration. Coppola et al. (7) suggested a modified, somewhat quicker method using a shorter ion exchange column but requiring fluorescamine derivatization before subsequent fluorometric detection. Conacher et al. (8) proposed a multi-step gas-liquid chromatographic method that requires anhydrous conditions for preparing the trimethylsilyl derivative of glutamic acid after an aqueous extraction and ion exchange purification.

The following procedure is a rapid, accurate HPLC method for determining MSG in food.

Received May 26, 1981. Accepted November 4, 1981.

METHOD

Apparatus and Reagents

(a) *HPLC system.*—Beckman Model 110A pump with 50 μL loop injector, Pharmacia RI monitor cooled to 20°C with a constant temperature ethylene glycol bath, and attached strip chart recorder. Whatman 25 cm × 4.6 mm id Partisil SAX column protected by 7 cm × 2.1 mm id guard column containing pellicular anion exchanger and, before the injector, a 25 cm × 4.6 mm id precolumn (Solvecon) containing silica gel.

(b) *Evaporator.*—Buchi Rotavapor-R with water bath maintained at ≤60°C.

(c) *Solvents.*—HPLC water was purified by reverse osmosis (Milli-RO) and further purified by using a Milli-Q system. All other solvents and reagents were reagent grade or better.

(d) *Buffer*—pH 4.0, 0.175M ammonium acetate. Dilute 20 mL glacial acetic acid to just under 2 L with HPLC water and adjust to pH 4.0 with 5M NH_4OH. Make up to 2 L with HPLC water.

(e) *Charcoal.*—Norit A (BDH Chemicals).

(f) *Celite.*—Celite 545 (Fisher Scientific).

(g) *Glutamic acid standard.*—Dissolve ca 50 mg, weighed to 0.1 mg, L-glutamic acid (A grade, Calbiochem-Behring Corp., PO Box 12087, San Diego, CA 92112) in 50 mL water. Prepare fresh daily.

(h) *Pyroglutamic acid standard.*—Dissolve ca 50 mg, weighed to 0.1 mg, D,L-pyroglutamic acid (Puriss, Koch-Light Laboratories Ltd, Poyle Estate

0004-5756/82/6503-0567-05$01.00

Willow Rd, Colnbrook, SL3 OBZ, Burkshire, UK) in 50 mL water. Prepare fresh daily.

(i) *Ion exchange resin.*—Dowex 50W-X8 acid form, 20–50 mesh (J.T. Baker Chemical Co.).

Glutamic Acid Determination

Extraction step is similar to the procedure of Fernandez-Flores et al. (6).

Into 150 mL beaker containing magnetic stir bar, weigh ca 5 g (to 1 mg) well mixed food sample and dilute to 35 mL with water. Stir 15 min, and then add 3 g charcoal and 30 mL acetone. Stir, let stand ca 15 min, and then filter through Celite pad. Wash beaker and residue with 75 mL acetone–water (1 + 1). Evaporate to small volume and dilute to exactly 50 mL with water. Inject 50 μL sample into HPLC system, alternating with injections of standard (**g**) (sometimes (**h**)). Chromatograph, with (**d**) as mobile phase, at flow rate of 1.5 mL/min. Identify peaks by retention time. Calculate glutamic acid (or pyroglutamic acid), using area comparison (peak height × width at half height) with standard (std) averaged before and after sample (sam).

% Glutamic acid = (area sam/averaged area std) × (wt std (mg)/10 × wt sam (g))

% MSG = % glutamic acid × 1.15

Acid Resin Purification

Fill 35 × 2.2 cm id column (fitted with stopcock) with 50 mL water, and pour in 15 g ion exchange resin with aid of another 50 mL water. Let water flow until it is level with top of resin. Take exactly 10 mL of the 50 mL food extract (from above determination). Adjust to between pH 1.5 and 2.0 with 1M HCl. Apply carefully (do not disturb resin) to column and again let this slowly penetrate until liquid is level with top of resin. Wash column with 50 mL water. Elute basic compounds (mainly amino acids) with 250 mL 5M NH_4OH. For first 25 mL eluate, run flow rapidly, but slow down to about 1 drop/s for last 225 mL. Evaporate this eluate to dryness. Add a few drops of formic acid and ca 10 mL water, and evaporate to dryness again. Take up in exactly 10 mL water.

Results and Discussion

Although the peak shape of glutamic acid varied somewhat with concentration, area measured as peak height times width at half height gave a linear response (Table 1). A practical limit for easy quantitation is about 0.1 mg glutamic acid/mL for most samples, using the above procedure or following modifications. For samples containing material that causes a sloping baseline (soy sauce), a value of 0.15 mg glutamic acid/mL is a more reasonable limit, although lower levels can be readily detected. Adjustments in the procedure could be made for samples high or low in glutamic acid. For Chicken-in-a-Mug (high in glutamic acid), a 1 g rather than a 5 g sample was taken. For bacon-flavored croutons (low in glutamic acid), the final sample was taken up in 25 mL rather than in 50 mL to further concentrate the glutamic acid.

Table 1. Area (average of 2 injections) found for glutamic acid at different concentrations (correlation coefficient = 0.9999)

Glutamic acid concn, mg/mL	Area, sq. mm	Area (sq. mm)/ concn (mg/mL)
3.078	2017	655
1.231	805	654
0.616	406	659
0.246	164	666
0.123	83	675

We felt that a dry sample low in glutamic acid would best test the extraction and recovery of glutamic acid. Because recovery using almost exactly the same extraction had been studied by other authors (6–8), we did not expect (or find) it to be a problem. Eight replicate determinations on bacon-flavored croutons gave an average of 0.144% glutamic acid (0.139, 0.147, 0.137, 0.146, 0.139, 0.151, 0.151, 0.144%) with a standard deviation of 0.0055%. Recovery of added glutamic acid averaged 99.2% (Table 2).

To check for compounds that might be expected to interfere with the glutamic acid peak, standards of several compounds were injected into the HPLC system. These included organic acids: citric, succinic, malic, lactic, quinic, shikimic, and pyroglutamic; other amino acids: aspartic acid, alanine, serine, lysine, and asparagine; inorganic compounds: salt and phos-

Table 2. Recovery of glutamic acid added to 5 g bacon-flavored croutons containing 7.2 mg glutamic acid (0.144%)

Added, mg	Total found, mg	Recovery, %
4.8	12.3	106
9.7	17.5	106
14.4	21.0	97.9
19	25.1	94.2
26.7	33.9	95.9
28	34.7	98.2
37.1	44.2	99.7
39.7	46.9	95.5

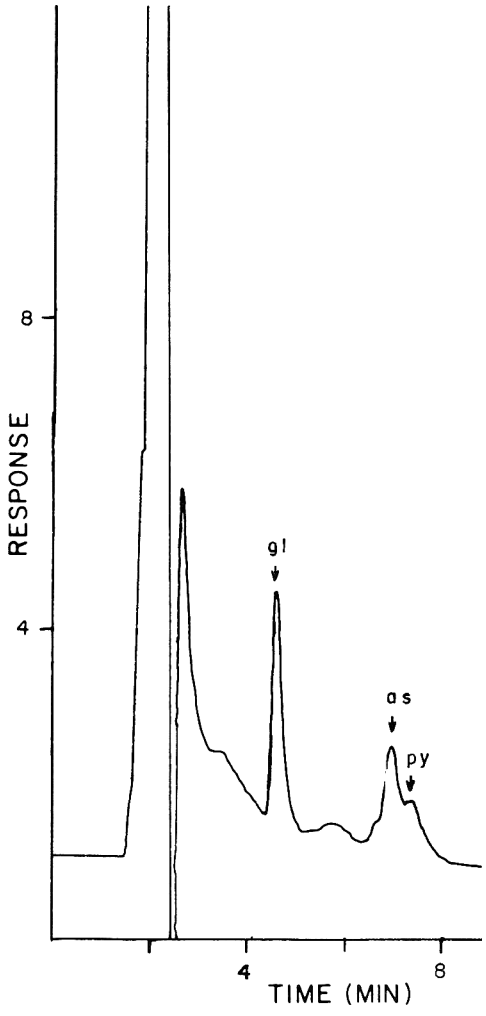


Figure 1. Chromatogram of soy sauce extract (0.93% glutamic acid). gl - glutamic acid; as = aspartic acid; py = pyroglutamic acid. Vertical scale: RI units × 10^5. Flow rate of buffer mobile phase 1.5 mL/min.

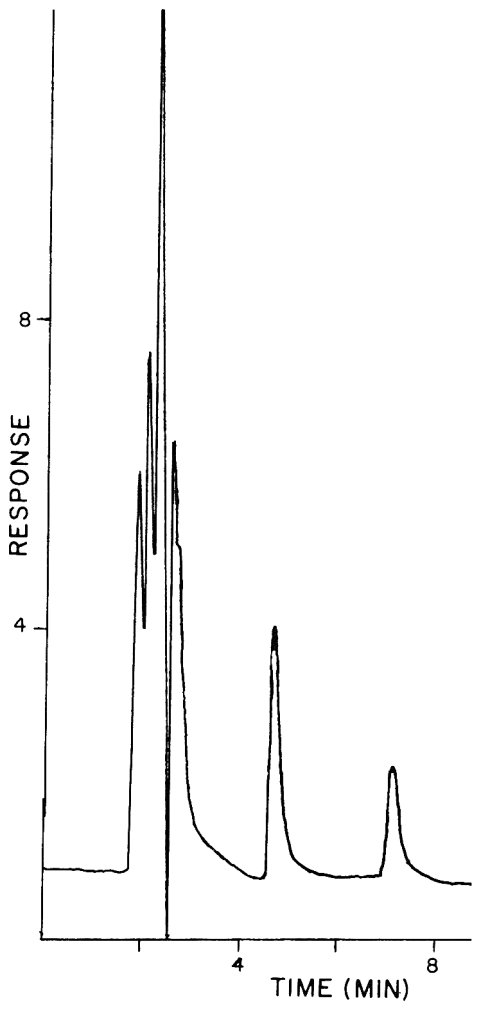


Figure 2. Chromatogram of soy sauce extract after acid resin purification (1.00% glutamic acid). Vertical scale: RI units × 10^5. Flow rate of buffer mobile phase 1.5 mL/min.

Table 3. Glutamic acid (%) found before and after acid resin purification

Food sample	Before purif.	After purif.
Chicken-in-a-Mug	6.85	6.85
Soy sauce	0.93	1.00
Won ton soup mix	1.86	1.80
Bacon-flavored croutons	0.14	0.15

phoric acid. None of these compounds interfered with the glutamic acid peak. All amino acids tested except aspartic acid eluted near or at the solvent front.

To further check for interfering compounds, 4 samples were analyzed for glutamic acid by the HPLC procedure, and then were purified on acid resin and again analyzed by the same procedure (Table 3 and Figures 1–3). This purification removes salts, sugars and other neutral compounds, and acids, leaving only basic compounds. The values for soy sauce (Table 3) are particularly significant because of the variety of possible interfering compounds in this food (9). To further check if any amino acids were interfering in the glutamic acid determination, the resin-purified soy sauce and won ton soup mix samples were chromatographed by a procedure

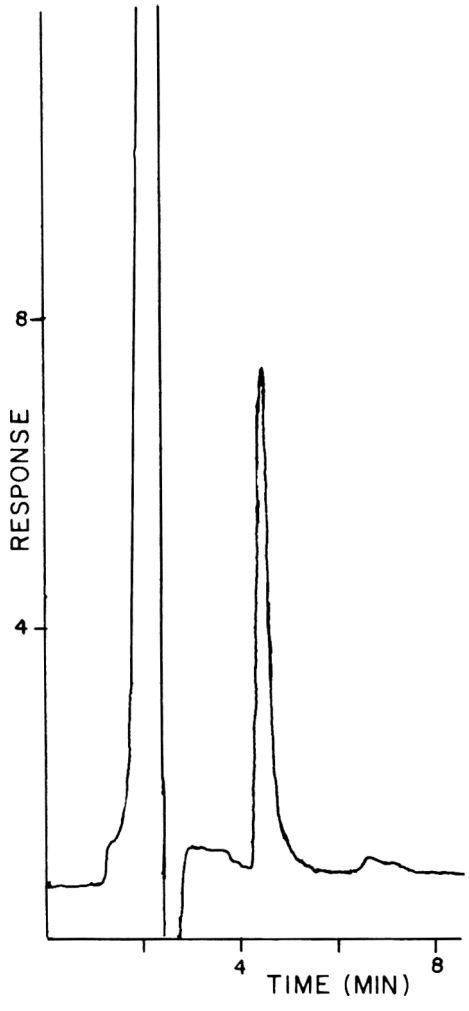


Figure 3. Chromatogram of won ton soup mix extract (1.86% glutamic acid). Vertical scale: RI units $\times 10^5$. Flow rate of buffer mobile phase 1.5 mL/min.

similar to that of Bailey and Swift (4). Instead of a 40 cm strip with ascending chromatography, a 50 cm strip and descending chromatography were used. By comparison with standards spotted at a large variety of concentrations, we estimated that the percentage of glutamic acid was ~1.0 for soy sauce and ~2.0 for the soup mix. These results agree well with values obtained using the HPLC method (Table 3).

Because the glutamic acid in soy sauce was already dissolved in water, a sample was prepared directly by diluting 5 g of soy sauce to 50 mL. This gave a slightly more colored solution (than by the extraction procedure) but on injection

Table 4. Area (average of 2 injections) found for pyroglutamic acid at different concentrations (correlation coefficient = 0.9999)

Pyroglutamic acid concn, mg/mL	Area, sq. mm	Area (sq. mm)/ concn (mg/mL)
2.947	1060	360
1.179	413	350
0.472	169	358
0.189	67	354

gave a trace virtually identical to Figure 1. This suggests that the glutamic acid determination in liquid samples could be made even faster, if necessary, by eliminating the extraction step entirely. This may necessitate cleaning the HPLC column more frequently. Because this was the only liquid sample examined, it is not certain if this shortened procedure can be applied to all liquid food samples.

Results for soy sauce also illustrate another advantage of the HPLC method. Pyroglutamic acid is well separated from glutamic acid (although it overlaps slightly with aspartic acid) (Figure 1). This means that this compound can also be detected in a glutamic acid determination. Pyroglutamic acid has also shown flavor enhancement (10), and causes off-flavor (11) in some food systems. None of the other methods of analysis for glutamic acid mentioned in this paper would detect the presence of pyroglutamic acid. In the cyclization of glutamic acid or glutamine to pyroglutamic acid, the basic nitrogen is lost, so pyroglutamic acid is ninhydrin-negative and is not retained by acid resins. All other glutamic acid analyses eliminate all non-basic compounds. Moreover, prolonged heating of a solution or mixture containing glutamine (12, 13) or the higher concentrations of glutamic acid found in flavor-enhanced foods could lead to an increase in pyroglutamic acid, which will most certainly change the food's flavor. With the HPLC method, such pyroglutamic acid production can be checked.

Pyroglutamic acid also gave a linear response for area (Table 4). The average recovery of pyroglutamic acid added to croutons was 104% (Table 5).

In foods which contain a large amount of aspartic acid, such as soy sauce (about 0.5% aspartic acid), accurate quantitation of pyroglutamic acid would be limited to levels of about 0.5 mg pyroglutamic acid/mL by the described procedures; however, in the majority of food samples analyzed using this method (with little or no aspartic

Table 5. Recovery of pyroglutamic acid added to bacon-flavored croutons

Added, mg	Found, mg	Recovery, %
10	11.6	116
22.3	21.9	98.2
30.0	30.5	102
37.9	38.1	101

acid, Figure 3) much lower levels of pyroglutamic acid could be quantitated.

Acknowledgment

Funding for this project from the General Research Grants Committee of the University of Alberta is gratefully acknowledged.

References

(1) *Chem. Eng. News* (1981) **59**(3), 28
(2) Schaumberg, H. H., Byck, R., Gerstl, R., & Mashman, J. H. (1969) *Science* **163,** 826–828
(3) *Food Technol.* (1980) **34**(10), 49–53
(4) Bailey, B. W., & Swift, H. L. (1970) *J. Assoc. Off. Anal. Chem.* **53,** 1268–1269
(5) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA secs **20.189-20.191**
(6) Fernandez-Flores, E., Johnson, A. R., & Blomquist, V. H. (1969) *J. Assoc. Off. Anal. Chem.* **52,** 744–746
(7) Coppola, E. D., Christie, S. N., & Hanna, J. G. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 58–61
(8) Conacher, H. B. S., Iyengar, J. R., Miles, W. F., & Botting, H. G. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 604–609
(9) Yokotsuka, T. (1960) in *Advances in Food Research,* C. O. Chichester & E. M. Mrak (Eds), Academic Press, New York, NY, pp. 75–134
(10) Gutzeit-Walz, R., & Solms, J. (1971) *Z. Lebensm. Unters. Forsch.* **146,** 86–90
(11) Maldi, A. A., Rice, A. C., & Weckel, K. G. (1961) *J. Agric. Food Chem.* **9,** 143–146
(12) Clydesdale, F. M., Lin, Y. D., & Francis, F. J. (1972) *J. Food Sci.* **37,** 45–47
(13) Acree, T. E., & Lee, C. Y. (1975) *J. Agric. Food Chem.* **23,** 828–830

High Pressure Liquid Chromatographic Determination of Glycyrrhizic Acid or Glycyrrhizic Acid Salts in Various Licorice Products: Collaborative Study

PETER S. VORA
MacAndrews & Forbes Co., Third St and Jefferson Ave, Camden, NJ 08104

Collaborators: H. Estes; Dr Grigor; B. Hodge; W. J. Hurst; D. LeBlanc; C. Shuford; K. Walker; M. Zimmermann

A collaborative study determining glycyrrhizic acid or glycyrrhizic acid salts content of various licorice products has been conducted using high pressure liquid chromatography (HPLC). Five samples containing various concentrations of glycyrrhizic acid were analyzed in blind replicates by 8 collaborators. The results indicate excellent repeatability and reproducibility with coefficients of variation less than 7.5%. In addition, this method allows the determination of glycyrrhizic acid in less than 15 min compared with 3 days for the conventional gravimetric and colorimetric methods. The method has been adopted official first action.

Glycyrrhizic acid or glycyrrhizic acid salt is one of the most important components of licorice products that determines their quality and cost. The current gravimetric and colorimetric methods (1, 2) are nonspecific, time-consuming, and not applicable to all licorice products. An accurate, simple, and rapid procedure for determining glycyrrhizic acid or glycyrrhizic acid salts by high pressure liquid chromatography (HPLC) (3) was reported in 1980. This paper is a report on the results of collaborative study of the method.

19. FLAVORS

The following liquid chromatographic method for the determination of glycyrrhizic acid or glycyrrhizic acid salts in licorice products was adopted official first action:

Glycyrrhizic Acid or Glycyrrhizic Acid Salts in Licorice

Liquid Chromatographic Method

Official First Action

19.C01 ***Apparatus***

(a) *Liquid chromatograph.*—With Model M6000A solv. delivery system, Model U6K universal injector, and Model 440 UV absorbance detector set at 254 nm, 0.2–0.1 AUFS range (Waters Associates, Inc.), or equiv.

(b) *Recorder.*—Strip chart 1- or 2-pen recorder (Houston Instrument Omni Scribe Model B5Z17-1, or equiv.). Chart speed 0.2 in./min.

(c) *Column.*—μBondapak C_{18}, 10 μm particle size, 30 cm × 4 mm id (Waters Associates, Inc.), or equiv.

(d) *Solvent and sample clarification kits.*—Org. and aq. (Waters Associates, Inc., or equiv.).

19.C02 ***Reagents***

(a) *Mobile phase.*—Use chromatgy grade reagents. H_2O-HOAc-CH_3CN (61 + 1 + 38). Filter and degas mobile phase with solv. clarification kit. Flow rate 2.0 mL/min.

(b) *Glycyrrhizic acid salt std soln.*—0.1 mg/mL. Dissolve 10 mg monoammonium glycyrrhizinate (available from MacAndrews & Forbes Co., Camden, NJ 08104) in 100 mL mobile phase. Filter thru 0.45 μm filter, using org. sample clarification kit. Prep. fresh std soln daily.

19.C03 ***Sample Preparation***

Dissolve 50 mg licorice product in 50 mL H_2O. Use mobile phase if not sol. in H_2O. Vortex-shake until sample is completely dissolved. Filter through 0.45 μm filter, using aq. sample clarification kit.

19.C04 ***Determination***

Warm up UV detector 15 min before start-up. Run mobile phase thru column at specified operating conditions ≥20 min before injecting first sample. Using 10 μL syringe, inject accurately measured vol. of std soln in duplicate. Inject similar vol. of sample soln. If samples are analyzed in series, re-inject std soln at regular intervals. Use peak ht (peak area in case of electronic integrator) to calc. concn of glycyrrhizic acid or glycyrrhizic acid salt as follows:

Received September 2, 1981. Accepted October 26, 1981.
This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.
The report 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.* (1982), March issue.

0004-5756/82/6503-0572-03$01.00

Glycyrrhizic acid monoammonium salt, %

$$= (C'/C) \times (PH/PH') \times (V'/V) \times 100$$

where C' and C = concn of std and sample soln in mg (dry basis)/mL, resp.; PH' and PH = peak ht of std and sample, resp.; V' and V = vol. of std and sample injected in μL, resp.

Results and Discussion

Samples of 5 licorice products (Ship SD, 'S' SD, Forbex BS-CS, ammonium glycyrrhizinate, and ammonium glycyrrhizinate CW) were sent to 8 laboratories. Samples were packed in plastic bags to minimize exposure. Samples were sent as blind duplicates and were numbered in a way that no participant analyzed the samples in the same sequence nor were any duplicates analyzed in sequence. Explicit instructions, a practice sample, standard sample, and reporting forms were provided. The standard sample was prepared by 3 additional crystallizations of commercially available monoammonium glycyrrhizinate from methanol and water.

The results of duplicate determinations of 5 samples by the HPLC method are presented in Table 1. Data were examined to see if any laboratory shows consistently high or low values. The sum of each replicate from Table 1 is shown ranked in Table 2. According to Duncan's range test (4), all laboratories were within the range, indicating no outlying laboratory. Dixon's test (4) to determine the outlying individual results indicated Laboratory 1 for Sample 3 and Laboratory 5 for Sample 1 did not satisfy the criterion. Statistical analyses have been carried out with and without these outlying individual results.

A one-way analysis of variance is used to compare precision within-laboratory and between-laboratories (Table 3).

Results indicate that the precision of the technique is excellent within-laboratory as indicated by repeatability standard deviation and coefficient of variation. Precision between laboratories is slightly lower, as expected; however, the reproducibility standard deviation and respective coefficient of variation of less than 7.5% indicate excellent precision. Variation in concentration of glycyrrhizic acid showed no significant effect on either test repeatability or reproducibility. Exclusion of 2 outlying individual results increased the precision of repeatability and reproducibility, but not significantly. Results obtained by collaborators matched well with known results obtained in our laboratory.

No major problems were indicated by collaborators. One collaborator requested a change in

Table 1. Collaborative data for HPLC determination of glycyrrhizic acid in licorice products

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
Coll.	Replicates	Sum	Replicates	Sum	Replicates	Sum	Replicates	Sum	Replicates	Sum
1	5.81 5.74	11.55	26.69 27.14	53.83	2.78 2.78	5.56	33.68 32.98	66.66	7.98 8.29	16.27
2	5.81 5.57	11.38	28.52 26.41	54.93	2.63 2.36	4.99	33.64 34.31	67.95	7.70 7.61	15.31
3	5.94 5.73	11.67	25.80 25.64	51.44	2.60 2.32	4.92	34.72 32.56	67.28	7.56 7.43	14.99
4	5.52 5.50	11.02	24.59 25.14	49.73	2.44 2.39	4.83	32.47 28.41	60.88	7.48 6.38	13.86
5	5.05 4.89	9.94	24.54 23.57	48.11	2.24 2.32	4.56	31.09 30.26	61.35	7.27 6.74	14.01
6	5.50 6.20	11.70	25.50 24.90	50.40	2.40 2.30	4.70	31.20 31.50	62.70	6.90 6.70	13.60
7	5.78 5.20	10.98	24.45 23.53	47.98	2.43 2.37	4.80	29.85 30.78	60.63	7.23 7.26	14.49
8	5.66 5.66	11.32	25.30 24.40	49.70	2.41 2.22	4.63	31.68 30.84	62.52	6.68 7.42	14.10

Table 2. Ranking of data from Table 1

Coll.	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Total rank
	Sum	Rank	Sum	Rank	Sum	Rank	Sum	Rank	Sum	Rank	
1	11.55	6	53.83	7	5.56[a]	8	66.66	6	16.27	8	35
2	11.38	5	54.93	8	4.99	7	67.95	8	15.31	7	35
3	11.67	7	51.44	6	4.92	6	67.28	7	14.99	6	32
4	11.02	3	49.73	4	4.83	5	60.88	2	13.86	2	16
5	9.94[a]	1	48.11	2	4.56	1	61.35	3	14.01	3	10
6	11.70	8	50.40	5	4.70	3	62.70	5	13.60	1	22
7	10.98	2	47.98	1	4.80	4	60.63	1	14.49	5	13
8	11.32	4	49.70	3	4.63	2	62.52	4	14.10	4	17

[a] Outlier (4).

Table 3. Statistical analysis of collaborative data

Statistic	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
	Including Outlying Individual Results				
Mean	5.598	25.383	2.437	31.873	7.289
Std dev.	0.1021	1.6338	0.0279	2.7923	0.2437
F-Ratio	2.751	6.382	3.713	3.036	2.903
Repeatability std dev.	0.2447	0.7045	0.1146	1.2359	0.3708
Coeff. of var., %	4.37	2.78	4.70	3.89	5.09
Reproducibility std dev.	0.3353	1.3534	0.1758	1.7556	0.5184
Coeff. of var., %	5.99	5.33	7.22	5.51	7.11
	Excluding Outlying Individual Results				
Mean	5.687	25.383	2.388	31.873	7.289
Std dev.	0.0515	1.6338	0.0126	2.7923	0.2437
F-Ratio	0.635	6.382	0.799	3.036	2.908
Repeatability std dev.	0.2582	0.7045	0.1225	1.2359	0.3708
Coeff. of var., %	4.54	2.78	5.13	3.89	5.09
Reproducibility std dev.	—	1.3534	—	1.7556	0.5184
Coeff. of var., %	4.54	5.33	5.13	5.51	7.11

the originally submitted results because of an error in calculation. The use of an internal standard was recommended by one collaborator; however, based on overall results, it may not be necessary. The use of reverse phase columns manufactured by various suppliers or use of electronic integrator vs. peak height measurement showed no significant effect on results.

Recommendation

Based on the excellent precision obtained within- and between-laboratories, with a coefficient of variation less than 7.5%, it is recommended that this method be adopted official first action.

Acknowledgments

The author thanks the following individuals who collaborated in this study:

Howard Estes, The American Tobacco Co., Hopewell, VA

Dr. Grigor, State Dept of Agriculture, Harrisburg, PA

Brenda Hodge, R. J. Reynolds Tobacco Co., Winston-Salem, NC

W. Jeffrey Hurst, Hershey Foods Technical Center, Hershey, PA

Dorrece LeBlanc, Lorillard, Greensboro, NC

Clinton Shuford, Liggett and Meyers Tobacco Co., Durham, NC

Ken Walker, Brown & Williamson Tobacco Co., Louisville, KY

Mike Zimmermann, Philip Morris USA, Richmond, VA

References

(1) Houseman, P. A. (1922) *J. Assoc. Off. Agric. Chem.* **6**, 191-196

(2) Cundiff, R. H. (1964) *Anal. Chem.* **36**, 1872-1873

(3) Vora, P. S. (1980) 94th Annual Meeting of the AOAC, Washington, DC, Abstr. 121

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

PESTICIDE FORMULATIONS

Evaluation of Silica and Polar Bonded Columns for Liquid Chromatographic Analysis of Temephos Formulations

DWIGHT L. MOUNT and JAMES W. MILES
Centers for Disease Control, Center for Infectious Diseases, Parasitic Diseases Division, Atlanta, GA 30333

In the course of development of an HPLC method for determination of temephos in technical grade temephos and its formulations, a variety of columns and solvent systems were evaluated. Three satisfactory methods evolved and were evaluated for reproducibility, convenience, cost, and optimum resolution of active ingredient from possible impurities. After consideration of advantages and disadvantages of each system, a method based on the use of a silica gel column eluted with ethyl acetate-hexane (10 + 90) and *p*-nitrophenyl *p*-nitrobenzoate as internal standard was selected for testing in an international collaborative trial.

Temephos (*O,O'*-(thiodi-4,1-phenylene)*bis*-(*O,O*-dimethyl phosphorothioate) was introduced in 1965 as a mosquito larvicide. The compound is widely used in the United States for mosquito control; it is the pesticide of choice for control of *Simulium damnosum* in the World Health Organization (WHO) Onchocerciasis Control Program in West Africa where it has been applied as a 20% emulsifiable concentrate since 1974 (1).

Methods of analysis for active ingredient content are required in specifications for the purchase of temephos formulations used in public health programs. In 1973, WHO adopted an ultraviolet absorption method (2) based on the work of Pasarela and Orloski (3) in which both ultraviolet and gas chromatographic methods were described. In the ultraviolet method, interfering substances were removed on a silica gel column. In the gas chromatographic method, cleanup was accomplished on an acidic alumina column. In 1979, WHO adopted a modified ultraviolet method in which the silica gel column was replaced by a silica gel thin layer plate (4).

Although these methods have been useful, it was considered that a high performance liquid chromatographic (HPLC) procedure might provide a faster and more precise analysis. In efforts to develop a suitable HPLC method, 4 HPLC columns and 2 solvent systems were tested for their ability to resolve temephos from impurities likely to be present in the product. Of the systems tested, 3 gave adequate resolution. A critical evaluation of the 3 selected systems was based on the degree of resolution, reproducibility, availability, and cost of the column and reagents as well as the convenience of the procedure.

Experimental

Apparatus and Reagents

(a) *Liquid chromatograph.*—Equipped with Varian Model 5000 pump; Valco loop injector; LKB UVicord Model 2138 detector with mercury discharge lamp, 254 nm filter, and flow cell with a 2.5 mm optical path length; and Varian Model CDS 111 data system.

(b) *Columns.*—(*1*) μPorasil (Waters Associates, Inc., Milford, MA): stainless steel, 300 × 3.9 mm id packed with 10 μm porous silica. (*2*) Partisil-10 (Whatman, Inc., Clifton, NJ): stainless steel, 250 × 4.6 mm id packed with 10 μm porous silica. (*3*) Micropak CN-10 (Varian Associates, Sunnyvale, CA): stainless steel, 300 × 4 mm id packed with alkylnitrile bonded to 10 μm LiChrosorb. (*4*) Micropak NH_2 (Varian Associates): stainless steel, 300 × 4 mm id packed with alkylamine bonded to 10 μm LiChrosorb.

(c) *n-Hexane.*—Non-spectro, distilled in glass (Burdick & Jackson Laboratories, Muskegon, MI). Dry over molecular sieve, 5Å, 8–12 mesh beads (Davison Chemical Co., Baltimore, MD) and filter through 0.45 μm Millipore filter (Millipore Corp., Bedford, MA).

(d) *Tetrahydrofuran (THF).*—UV grade (Burdick & Jackson Laboratories). Pack lower part

These studies were conducted as part of a contractual agreement between the Centers for Disease Control and the World Health Organization.
Presented in part at the 181st meeting of the American Chemical Society, Atlanta, GA, April 1981.
Use of trade names or commercial sources does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.
Received September 15, 1981. Accepted December 4, 1981.

0004-5756/82/6503-0575-05$01.00

of 75 × 2.5 cm id glass column with 100 g alumina, neutral, Brockman Activity I, 80–200 mesh, dried at 250°C; pack upper part with 100 g silica gel, grade 950, 80–200 mesh, dried at 175°C. Fit column into 2-hole stopper and place in 500 mL flask containing 100 g 5Å molecular sieve (8–120 mesh beads) dried at 250°C. Place drying tube in other hole of stopper. Pass THF through column. Filter THF through Millipore filter and store under nitrogen.

(e) *Ethyl acetate.*—(Burdick & Jackson Laboratories). Dry over molecular sieve, 5Å, 8–12 mesh beads.

(f) *Anthracene methanol.*—Aldrich Chemical Co., Inc., Milwaukee, WI.

(g) *p-Nitrophenyl p-nitrobenzoate (NPNB).*—Prepare by reacting *p*-nitrobenzoyl chloride (Aldrich Chemical Co.) with an excess of *p*-nitrophenol sodium salt (Eastman Kodak Co., Rochester, NY) in acetonitrile.

(h) *Bis(p-chlorophenyl) sulfone.*—Aldrich Chemical Co.

(i) *Standards.*—Temephos, 99.6% (American Cyanamid Co., Princeton, NJ).

(j) *Impurities.*—See below.

1. $(CH_3O)_2P(S)O$⟨○⟩
2. $(CH_3O)_2P(S)O$⟨○⟩Cl
3. $(CH_3O)_2P(S)O$⟨○⟩SCH_3
4. $(CH_3O)_2P(S)O$⟨○⟩S⟨○⟩OCH_3
5. $(CH_3O)_2P(S)O$⟨○⟩$S—S$⟨○⟩$OP(S)(OCH_3)_2$
6. $(CH_3O)(CH_3S)P(O)O$⟨○⟩S⟨○⟩$OP(O)(OCH_3)(SCH_3)$

Determination of Resolution

For each of the experimental systems, the resolution of temephos from likely impurities was determined by injecting a mixture of pure temephos and impurities into the liquid chromatograph. Structures of these impurities are shown above. All impurities except *O,O*-dimethyl *O*-*p*-thiomethylphenyl phosphorothioate were furnished by the American Cyanamid Co. This latter compound was synthesized by first reacting 4-(methylmercapto)phenol (Aldrich Chemical Co.) with sodium hydride (50% dispersion in mineral oil, Aldrich Chemical Co.) in acetonitrile to form the sodium salt. This salt was reacted with *O,O*-dimethyl chlorophosphorothioate (Aldrich Chemical Co.) in acetonitrile to give the desired product. With the exception of the disulfide analog, all impurities were easily separated from temephos by each of the systems tested. The *S*-methyl isomer of temephos (structure 6, above) did not elute from any of the columns tested.

Resolution of temephos from the disulfide analog was determined by injecting a mixture of comparable concentrations of each of the 2 compounds into the various systems. *R*-values were calculated according to the formula $R = 2\Delta t/(W_1 + W_2)$, where t = difference in retention times of the compounds, and W_1 and W_2 are peak widths in units of time measurement at their bases.

Method I: Silica Column Eluted with THF-Hexane

Internal standard solution.—Weigh 0.2 g anthracene methanol into 250 mL volumetric flask and dilute to volume with treated THF.

Standard and sample solutions.—Accurately weigh quantity of material equal to ca 60 mg active ingredient into 50 mL volumetric flask. Add, by pipet, 5 mL internal standard solution and 15 mL treated THF. Shake flask to ensure dissolution of temephos, and dilute to volume with *n*-hexane.

Mobile phase.—Add, by pipet, 150 mL treated THF to 1 L volumetric flask and dilute to volume with dry *n*-hexane.

Column.—Install silica gel column. To render column essentially dry, pump 50 mL anhydrous methanol through column, followed by 100 mL treated THF. After this treatment, pump sufficient mobile phase through column to equilibrate system.

Liquid chromatography conditions.—Column temperature ambient, flow rate 1.0 mL/min, detector sensitivity 1.0 AUFS, injection volume 10 μL, retention times: temephos 10.6 min, internal standard 14.6 min.

Calibration.—Calibrate system by injecting 10 μL aliquots of standard solution until relative factors agree ±2%. When this requirement is met, system is ready for analysis of samples.

Analysis.—Analyze unknown samples by injecting 10 μL aliquots of sample solutions and measuring areas of internal standard and temephos peaks. Quantitate unknowns by comparison of relative factors of unknown and standard solutions.

Method II: Silica Column Eluted with Ethyl Acetate–Hexane

Internal standard solution.—1.5 g *p*-nitrophenyl *p*-nitrobenzoate/250 ml dry ethyl acetate.

Standard and sample solutions.—Accurately weigh quantity of material equal to ca 60 mg active ingredient into 50 mL volumetric flask. Add, by pipet, 5 mL internal standard solution and 25 mL dry ethyl acetate. Shake flask to ensure dissolution of temephos and dilute to volume with dry *n*-hexane.

Mobile phase.—Add, by pipet, 100 mL dry ethyl acetate to 1 L volumetric flask and dilute to volume with dry *n*-hexane.

Column.—Install silica gel column. To render column essentially dry, pump 50 mL anhydrous methanol through column followed by 100 mL dry ethyl acetate. After this treatment, pump sufficient mobile phase through column to equilibrate system.

Liquid chromatography conditions.—Column temperature ambient, flow rate 1.0 mL/min (1.5 mL/min for Partisil-10 column), detector sensitivity 1.0 AUFS, injection volume 10 μL, retention times: internal standard 11.0 min, temephos 13.3 min.

Calibration.—Same as Method I.

Analysis.—Same as Method I.

Method III: Alkylamine Bonded-Phase Column Eluted with Ethyl Acetate–Hexane

Internal standard solution.—Weigh 1.5 g bis(*p*-chlorophenyl) sulfone into 250 mL volumetric flask and dilute to volume with dry ethyl acetate.

Standard and sample solutions.—Accurately weigh quantity of material equal to ca 60 mg active ingredient into 50 mL volumetric flask. Add, by pipet, 5 mL internal standard solution and 25 mL dry ethyl acetate. Shake flask to ensure dissolution of temephos and dilute to volume with *n*-hexane.

Mobile phase.—Add, by pipet, 150 mL dry ethyl acetate to 1 L volumetric flask and dilute to volume with dry *n*-hexane.

Column.—Install alkylamine bonded-phase column. To render column essentially dry, pump 50 mL anhydrous methanol through column followed by 100 mL dry ethyl acetate. After this treatment, pump sufficient mobile phase through column to equilibrate the system.

Liquid chromatography conditions.—Column temperature ambient, flow rate 1.0 mL/min, detector sensitivity 1.0 AUFS, injection volume 10 μL, retention times: internal standard 12.7 min, temephos 16.9 min.

Calibration.—Same as Method I.

Analysis.—Same as Method I.

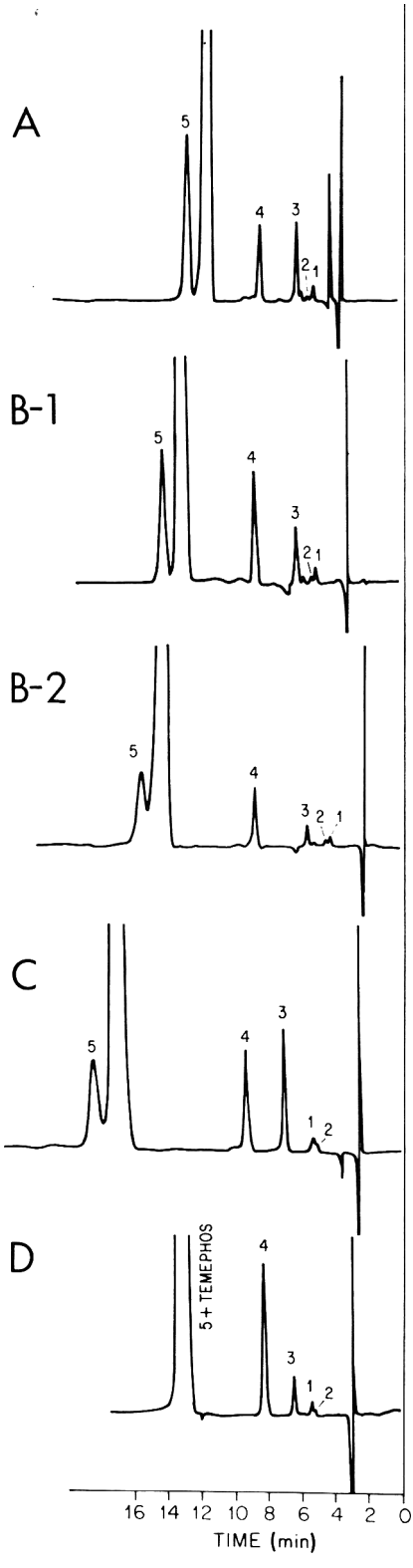
Results and Discussion

Data obtained on a sample of technical grade temephos, using the 3 different methods, are presented in Table 1. The relative factors obtained on the internal standard solutions used in the analyses were calculated by multiplying the weight of temephos in the solution (mg) by the area of the internal standard peak divided by the area of the temephos peak. Standard solutions were injected in replicate on different days using

Table 1. Results of analysis of temephos, technical, obtained with 3 different HPLC systems

Method	Std soln rel. factors		Active ingredient found, %		Resolution (R)[a]
	Av. ± SD	Rel. SD, %	Av. ± SD	Rel. SD, %	
I. Silica column, μPorasil; THF–hexane (15 + 85)	$\bar{X}_3$ = 84.40 ± 0.26	0.31	$\bar{X}_4$ = 86.57 ± 0.07	0.08	
	$\bar{X}_6$ = 82.56 ± 0.27	0.33	$\bar{X}_4$ = 86.04 ± 0.92	1.07	
	$\bar{X}_5$ = 81.51 ± 0.07	0.09			1.9
	$\bar{X}_4$ = 92.2 ± 1.80	1.95			
	$\bar{X}_4$ = 102.07 ± 1.77	1.73			
II. A. Silica column, μPorasil; ethyl acetate–hexane (10 + 90)	$\bar{X}_4$ = 66.44 ± 0.16	0.24	$\bar{X}_4$ = 86.16 ± 0.32	0.37	
	$\bar{X}_4$ = 66.09 ± 0.36	0.54			1.5
II. B. Silica column, Partisil-10; ethyl acetate–hexane (10 + 90)	$\bar{X}_4$ = 66.63 ± 0.22	0.33	$\bar{X}_4$ = 86.14 ± 0.24	0.28	
	$\bar{X}_4$ = 66.40 ± 0.40	0.60			1.5
III. Alkylamine bonded-phase column, Micropak NH_2; ethyl acetate–hexane (15 + 85)	$\bar{X}_4$ = 62.58 ± 0.20	0.32	$\bar{X}_4$ = 86.12 ± 0.35	0.41	
	$\bar{X}_4$ = 62.22 ± 0.19	0.31			1.3

[a] Resolution of temephos and temephos disulfide = $R = 2\,\Delta t\,(W_2 + W_1)$.



different preparations of mobile phase solution to obtain the data presented in the second column of the table. The average values for active ingredient were calculated from replicate values obtained on separate weighings of the sample. The R-values for the resolution of temephos and the disulfide analog were calculated as described in the text.

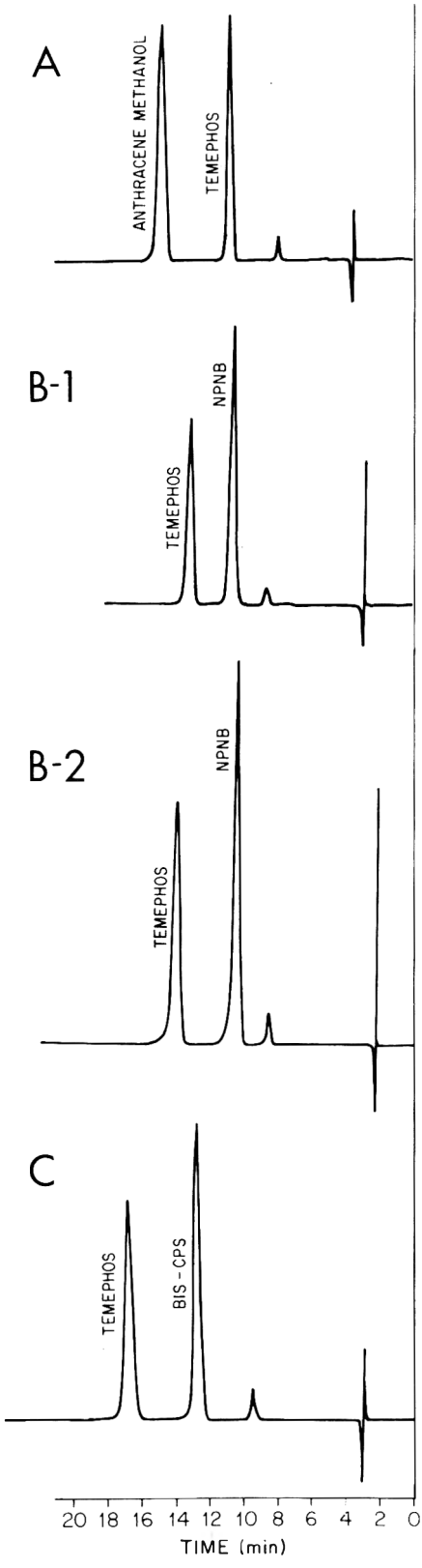
Chromatograms of a mixed standard (temephos plus the 6 impurities) obtained on each of the systems are presented in Figure 1. Those of the technical product obtained with each of the 3 methods are presented in Figure 2.

Evaluation of Method I.—Of the 3 methods, this method gave the best resolution between temephos and its disulfide analog (R = 1.9). However, reproducibility of the method was dependent on the quality of THF with respect to the presence of peroxides. Temephos was very sensitive to oxidation by peroxides when injected onto a silica column. Thus, the response factor for temephos depends on the amount of peroxides present at the time of injection. The reproducibility of the system was acceptable (although not as good as for the other methods) when freshly prepared THF was used in preparing the mobile phase. Severe problems with reproducibility were observed when week-old THF was used, even though it had been stored under nitrogen. In addition, the retention time (t_R) of the internal standard (anthracene methanol) was very sensitive to the activity of the column. However, this presented no major problem in the analyses once the system had stabilized.

Evaluation of Method II.—Two different silica columns, μPorasil and Partisil-10, were evaluated for use in this method. The resolution of the temephos and disulfide peaks was calculated to be the same for both columns (R = 1.5). However, due to a larger tailing effect observed for the Partisil-10 column, the μPorasil column appears to give a better separation of temephos from the disulfide analog (see Figure 1). From Table 1 it can be seen that reproducible results were obtained on both columns and both are

Figure 1. HPLC traces of a mixture of temephos and 6 possible impurities.

A, μPorasil eluted with THF–hexane; B-1, μPorasil eluted with ethyl acetate–hexane; B-2, Partisil-10 eluted with ethyl acetate–hexane; C, MicroPak NH_2 eluted with ethyl acetate–hexane; D, MicroPak CN-10 eluted with ethyl acetate–hexane. For peak identity see structures.



recommended for use in this system. A disadvantage of this system is that the internal standard, *p*-nitrophenyl *p*-nitrobenzoate, is not commercially available and must be synthesized. However, the stability and reproducibility of this system and the convenience of preparing the mobile phase, as compared with that in Method I, far outweigh the minor inconvenience of synthesizing the internal standard.

Evaluation of Method III.—This method was developed to compare the performance of polar-bonded-phase columns and silica. A MicroPak CN-10 column was first considered. This column failed to separate temephos from the disulfide analog and, therefore, was abandoned early. The MicroPak NH_2 column separated these 2 compounds adequately (R = 1.3), although not quite as well as the silica column. The amino column gave results comparable to the silica column in terms of reproducibility; however, for some unexplained reason, a slow systematic shift of retention times of the eluants was observed in this system. The magnitude of the shift was observed to be about 1–1.5 min over a period of 3–4 h. This presented no major problems in obtaining good results. An advantage of this method over Method II is the commercial availability of the internal standard, bis(*p*-chlorophenyl) sulfone.

In view of the stability, reproducibility, convenience, and universal availability and acceptability of silica columns, Method II was the method of choice for the determination of temephos in technical and formulated products. This method was selected for testing in an international collaborative study.

References

(1) Walsh, J. F., Davies, J. B., & LeBerre, R. (1979) *Tropenmed. Parasitol.* **30**, 328–344
(2) *Specifications for Pesticides Used in Public Health* (1973) 4th Ed., World Health Organization, Geneva, Switzerland
(3) Pasarela, N. R., & Orloski, E. J. (1973) in *Analytical Methods for Pesticides and Plant Growth Regulators*, Vol. VII. J. Sherma and G. Zweig (Eds), Academic Press, New York, NY, pp. 119–146
(4) *Specifications for Pesticides Used in Public Health* (1979) 5th Ed., World Health Organization, Geneva, Switzerland

Figure 2. HPLC traces of mixture of technical grade temephos and internal standards.

A, μPorasil eluted with THF–hexane; B-1, μPorasil eluted with ethyl acetate–hexane; B-2, Partisil-10 eluted with ethyl acetate–hexane; C, MicroPak NH_2 eluted with ethyl acetate–hexane.

High Performance Liquid Chromatographic Method for Determination of Temephos in Technical and Formulated Products: Collaborative Study

JAMES W. MILES and DWIGHT L. MOUNT
Centers for Disease Control, Center for Infectious Diseases, Parasitic Diseases Division, Atlanta, GA 30333

Collaborators: J. B. Audino; A. A. Carlstrom; C. S. Crisp; R. L. Denning; J. Gardiner; G. M. Gentry; P. Hitos; W. E. Hodgins; P. Jung; W. King; M. W. Law; R. Lopata; J. P. Minyard; K. Paval; S. E. Rivera; N. E. Skelly; E. V. Sorensen; M. Vaysse; R. S. Wayne; G. R. Winstead III; D. Wong

An HPLC method for the determination of temephos in temephos technical and formulated products has been subjected to an international collaborative study with 14 laboratories participating. Samples were extracted with ethyl acetate and eluted on a silica gel column with ethyl acetate-hexane (1 + 9); *p*-nitrophenyl *p*-nitrobenzoate served as the internal standard. Collaborators were furnished samples of technical, 20 and 50% emulsifiable concentrates, 50% water-dispersible powder, and 1% sand granules. The coefficients of variation of the values obtained on the 5 samples were 1.21, 2.02, 1.26, 1.89, and 9.90%, respectively. The method has been adopted official first action.

Temephos is available in a variety of formulations including water-dispersible powders, emulsifiable concentrates, and granules. In view of the increased interest in temephos in both agriculture and public health and the lack of a convenient method for analysis of temephos formulations, Mount and Miles (1) studied high pressure liquid chromatographic (HPLC) systems which could be used for determining temephos. Of 4 systems tested, a method based on the use of a silica gel column eluted with ethyl acetate-hexane was best suited for this purpose. Three laboratories subjected the method to a preliminary collaborative trial. Results of this trial were presented at the 25th Annual Meeting of the Collaborative International Pesticides Analytical Council (CIPAC) by Miles and Mount (2).

The method with minor modifications was then submitted to 14 collaborating laboratories including 5 from CIPAC member countries. Samples of temephos technical, 50% emulsifiable concentrate, 50% water-dispersible powder, and 1% sand granules were furnished by American Cyanamid Co., Princeton, NJ. A sample of 20% emulsifiable concentrate was furnished by Procida, Groupe Roussel UCLAF, Marseilles, France. Each collaborator was furnished with a subsample of the technical material and each of the formulations along with purified reference standard temephos and the internal standard. Collaborators were requested to weigh 2 aliquots of each sample and analyze each aliquot in duplicate. The experiment was designed according to suggestions given by Youden and Steiner (3).

Temephos (*O,O'*-(Thiodi-4,1-phenylene)-bis(*O,O*-dimethyl Phosphorothioate)

Official First Action

CIPAC-AOAC High Pressure Liquid Chromatographic Method

(Method is suitable for tech. temephos and formulations with temephos as only active ingredient.)

Principle

Sample is dissolved in ethyl acetate, *p*-nitrophenyl *p*-nitrobenzoate is added as internal std and, after diln with *n*-hexane, sample is injected into liq. chromatgc column. HPLC response ratio of insecticide to internal std is compared with response ratio of std to give temephos content in sample.

Apparatus and Reagents

(a) *Liquid chromatograph.*—Able to generate >2000 psi and measure *A* at 254 nm.

(b) *Chromatographic column.*—Stainless steel, 300 × 3.9 mm id packed with 10 μm silica gel (μ-Porasil, Waters Associates, Inc., is suitable.)

Use of trade names or commercial sources does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

Received August 28, 1981. Accepted October 26, 1981.
This report was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.
The report was approved by the General Referee and Committee A and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1982) **65**, March issue.

0004-5756/82/6503-0580-04$01.00

(c) *Ethyl acetate.*—Burdick & Jackson Laboratories, Inc. Dry over molecular sieve, 5Å, 8–12 mesh beads (Davison Chemical Co., Baltimore, MD). Filter through 0.45 μm Millipore filter (Millipore Corp., Bedford, MA 01730).

(d) *n-Hexane.*—Non-spectro, distd in glass (Burdick & Jackson Laboratories, Inc.). Dry over molecular sieve, 5Å, 8–12 mesh, and filter through 0.45 μm Millipore filter.

(e) *p-Nitrophenyl p-nitrobenzoate internal std.*—1.5 g/250 mL ethyl acetate. React *p*-nitrobenzoyl chloride (Aldrich Chemical Co.) with excess *p*-nitrophenol sodium salt (Eastman Kodak Co.) in CH_3CN. Alternatively, prep. 1.1% (w/v) dimethyl 4-nitrophthlate in ethyl acetate.

(f) *Reference std soln.*—Accurately weigh ca 50, 60, and 70 mg temephos, anal. reagent (American Cyanamid Co.) into sep. 50 mL vol. flasks. Add by pipet 5 mL internal std soln and 25 mL dry ethyl acetate to each flask. Shake flasks to ensure dissolution of std, and dil. to vol. with *n*-hexane. Designate solns as A, B, and C. Use soln B as working std soln for liq. chromatgy; use solns A and C to check linearity of liq. chromatograph (see *Linearity Check*) and to guard against weighing error in prepn of std soln. Supply of soln B can be replenished from time to time without prepg new supplies of solns A and C, provided linearity requirement described under *Linearity Check* can be met.

(g) *LC mobile phase.*—Add 100 mL dry ethyl acetate to 1 L vol. flask and dil. to vol. with dry *n*-hexane.

(h) *HPLC operating conditions.*—Column temp. ambient; flow rate 1.0 mL/min (ca 450 psi); retention times: internal std ca 9.6 min, temephos ca 11.5 min. Pump 50 mL anhyd. MeOH thru column followed by 100 mL dry ethyl acetate. Pump LC mobile phase thru column until system is equilibrated (flat baseline). Inject 5 μL aliquots of std soln B until const. response is obtained. If necessary, adjust instrument or injection vol. (usually 3–6 μL) to give 50–60% FSD for internal std peak. Use same injection vol. and instrument settings for all samples and stds.

Linearity Check

Inject triplicate aliquots of appropriate vol. (as detd above) of std solns A, B, and C into liq. chromatograph, det. response ratio for each injection, and av. resulting ratios for each soln. Divide av. response ratio for each soln by corresponding content (in mg) and compare resulting response factors. These factors should agree within 2%.

Liq. chromatograph should be checked for linearity at least once a week, and same check should be carried out whenever new std solns are prepd and whenever column, new or used, is installed in instrument.

Sample Preparation

(a) *Technical and emulsifiable concentrates.*—Accurately weigh amt sample contg ca 60 mg temephos directly into tared 50 mL vol. flask. For temephos tech., warm and thoroly mix before sampling. Add by pipet exactly 5 mL internal std soln and 25 mL dry ethyl acetate. Shake flask to ensure dissolution and dil. to vol. with *n*-hexane.

(b) *Water-dispersible powders and sand granules.*—Accurately weigh amt sample contg ca 60 mg temephos directly into 2 oz bottle fitted with plastic screw cap. Add by pipet 5 mL internal std soln and 25 mL dry ethyl acetate and shake 1 min. Add 20 mL *n*-hexane, mix thoroly, and let particles settle. Filter portion of soln and hold for HPLC analysis. (In some cases, centrifugation may be sufficient to remove particles before HPLC analysis.)

Analysis of Sample Solutions

Inject duplicate aliquots of std soln. B. Calc. response ratios by dividing area (or ht) of temephos peak by that of internal std peak. Response ratios should agree within 2%. Average duplicate response ratios obtained with std solns.

Inject duplicate aliquots of each sample soln. Average duplicate response ratios for each sample soln. Note: After first injection of any sample, let instrument run ≥30 min after emergence of temephos peak to det. late-eluting peaks due to impurities. Subsequent injections should be timed so that late-eluting peaks from sample injections do not interfere with internal std or temephos peaks of subsequent samples.

Inject duplicate aliquots of std soln B. Average response ratios of stds immediately before and after sample solns, which should agree within 2%. Use this av. to calc. temephos content of sample solns.

Calculations

For each injection, response ratio (R) = (area temephos peak/area internal std peak).

Temephos, wt% = $(R \times W' \times P)/(R' \times W)$ where R' and R = average response ratio for std soln B and sample soln, resp.; W' and W = wt (mg) of temephos std taken (for std soln B) and sample, resp; and P = purity of temephos std (%).

Table 1. Data obtained in collaborative trial of HPLC method for analyzing temephos technical and formulations

	Technical		20% EC		50% EC		50% WDP		1% SG	
Coll.	1	2	1	2	1	2	1	2	1	2
1	94.17	95.06	22.04	22.01	48.84	49.05	48.91	48.85	1.00	1.00
	94.72	95.49	21.94	21.92	48.18	48.90	49.03	48.93	1.01	1.01
Av.	94.44	95.27	21.99	21.96	48.51	48.97	48.97	48.89	1.00	1.00
2	95.55	94.22	21.72	22.08	50.81[a]	49.90	47.89	48.70	1.02	0.98
	93.04	93.94	21.61	21.37	50.02	50.03	48.48	48.82	0.99	1.01
Av.	94.29	94.08	21.66	21.72	50.41	49.96	48.18	48.76	1.01	0.99
3	93.78	93.38	21.87	22.04	49.48	49.19	49.16	49.72	1.03	0.99
	94.37	93.18	21.79	22.01	49.48	48.96	49.16	49.47	1.04	1.00
Av.	94.07	93.28	21.83	22.02	49.48	49.07	49.16	49.60	1.03	0.99
4	92.50	92.95	20.72[a]	21.13	48.86	48.79	49.66	49.30	1.08	1.12
	91.96	92.60	20.68[a]	21.07	49.39	48.75	49.61	49.47	1.08	1.13
Av.	92.23	92.77	20.70	21.10	49.13	48.77	49.63	49.38	1.08	1.13
5	94.14	93.70	21.71	21.60	48.97	49.92	50.72	50.23	0.95	1.08
	93.86	93.86	21.83	21.52	49.30	50.70[a]	50.95[a]	49.89	0.97	1.10
Av.	94.00	93.78	21.77	21.56	49.13	50.31	50.83	50.06	0.96	1.09
6	85.10[a]	85.10[a]	21.30	21.10	48.00	47.80	48.10	47.60	1.00	1.09
	85.20[a]	85.40[a]	21.30	21.10	47.90	47.70	48.10	47.50	1.01	1.09
Av.	85.15	85.25	21.30	21.10	47.95	47.75	48.10	47.55	1.01	1.09
7	92.50	93.60	21.60	21.60	48.20	48.20	49.20	48.90	1.22	1.22
	93.20	94.10	21.70	21.70	48.20	48.20	49.30	48.90	1.24	1.23
Av.	92.85	93.85	21.65	21.65	48.20	48.20	49.25	48.90	1.23	1.22
8	94.87	94.76	21.59	21.69	49.53	50.77[a]	49.10	47.13	1.11	1.10
	94.32	94.18	21.66	21.53	48.32	48.98	47.23	47.16	1.10	1.10
Av.	94.60	94.47	21.63	21.61	48.93	49.88	48.16	47.15	1.10	1.10
9	94.33	93.98	21.98	22.00	49.11	48.96	49.86	49.98	0.94	1.07
	94.40	94.04	21.90	21.93	49.06	48.90	49.88	49.96	0.94	1.06
Av.	94.36	94.01	21.94	21.96	49.08	48.93	49.87	49.97	0 94	1.07
10	96.14	95.10	22.36	22.48	49.69	49.87	49.48	50.19	1 25	1.27
	96.55	95.43	22.31	22.37	49.60	49.96	49.83	49 86	1 25	1.27
Av.	96.35	95.26	22.33	22.42	49.65	49.91	49.65	50 02	1.25	1.27
11	93.50	93.50	21.60	21.30	49.40	49.90	48.10	48.00	1.23	1.24
	93.20	93.80	21.40	21.40	49.70	50.00	48.10	49.00	1.23	1.22
Av.	93.25	93.65	21.50	21.35	49.55	49.95	48.10	48.50	1.23	1.23
12	93.80	93.80	22.60	22.60	48.40	48.80	50.00	49.80	1.01	1.00
	94.00	93.80	22.70	22.70	48.20	49.00	49.80	49.70	1.01	0.90
Av.	93.90	93.80	22.65	22.65	48.30	48.90	49.90	49.75	1.01	0.95
13	92.20	91.07	21.93	22.05	49.66	49.57	47.97	48.79	1.08	0.97
	92.30	91.29	21.95	21.76	49.76	49.80	48.02	48.59	1.08	0.97
Av.	92.25	91.18	21.94	21.91	49.71	49.69	48.00	48.69	1.08	0.97
14	92.99	93.02	22.86	22.95[a]	49.14	48.94	47.20	46.78[a]	1.28	1.20
	92.85	92.86	22.93[a]	22.99[a]	49.16	48.98	46.79[a]	47.14	1.29	1.20
Av.	92.92	92.94	22.90	22.97	49.15	48.96	47.00	46.96	1.28	1.20
N	52		51		52		53		56	
Mean	93.77		21.83		49.09		48.93		1.090	
SD	1.13		0.44		0.62		0.93		0.108	
CV, %	1.21		2.02		1.26		1.89		9.90	

[a] Observations outside 2 SD (95% confidence interval) omitted from calculations as described by Dixon and Massey (4).

Results and Discussion

A complete set of data was received from each of the 14 collaborators (Table 1). No serious problems were reported by any of the collaborators. One collaborator substituted isooctane for hexane in the eluting solvent; another substituted heptane. One collaborator diluted standard and sample solutions 1:10 before injection. One failed to dry the column with dry methanol before analysis of the samples. One dried the solvents over sodium sulfate rather than molecular sieve.

Participants used 10 brands of pumps, 8 brands of injectors, 7 brands of columns, and 8 brands of detectors in conducting their analyses. Eleven participants used irregular silica gel, whereas 3 used spherical silica gel. Ten used variable wavelength detectors and 4 used filters; 5 used an Hg source and 9 used a D_2 source. Cell paths of the detectors varied from 2.5 to 10 mm, volume of sample injected varied from 5 to 20 μL, pressures varied from 200 to 1600 psi, and flow rates varied from 1.0 to 1.5 mL/min.

Eleven participants determined response ratios with electronic integrators to measure peak areas while 3 participants used peak height measurements.

Excellent agreement among the collaborating laboratories was obtained on the technical grade sample and all formulations except the 1% sand granules. The coefficient of variation for this sample was 9.9%. It is our opinion that the nonhomogeneity of this sample contributed to the relatively large variance observed. The coefficients of variation for the other samples ranged from 1.21 to 2.0%, after eliminating all values outside the 95% confidence interval.

One disadvantage of the proposed method is the lack of a commercial source of the internal standard *p*-nitrophenyl *p*-nitrobenzoate, although it can be easily prepared in the laboratory (1). Subsequent to the completion of the collaborative trial, dimethyl 4-nitrophthalate was found to serve satisfactorily as an internal standard. Under the chromatographic conditions described above, this internal standard emerges in about 18 min as compared with 9.6 min for *p*-nitrophenyl *p*-nitrobenzoate.

The HPLC method was adopted for temephos technical, water-dispersible powders, and emulsifiable concentrates by CIPAC at its 25th Annual Meeting in Gembloux, Belgium. It is recommended that the method be adopted official first action by AOAC.

Acknowledgments

The authors thank Alan Huong for assistance in providing a statistical analysis of the data, and the following collaborators and their associates for their cooperation in this study:

J. B. Audino and S. E. Rivera, State Dept of Food and Agriculture, Sacramento, CA

A. A. Carlstrom and Diane Wong, Chevron Chemical Co., Richmond, CA

C. S. Crisp and J. Gardiner, Dept of Services and Supply, Adelaide, South Australia

G. M. Gentry and Wendy King, State Dept of Agriculture and Consumer Services, Tallahassee, FL

Pilar Hitos, Ministerio de Agricultura, Madrid, Spain

Paul Jung and Mark W. Law, U.S. Environmental Protection Agency, Beltsville, MD

J. P. Minyard and W. E. Hodgins, State Chemical Laboratory, Mississippi State, MS

K. Paval, Bayer AG, Wuppertal, GFR

N. E. Skelly, Dow Chemical, Midland, MI

E. V. Sorensen, A/S Cheminova, Lemvig, Denmark

M. Vaysse, Procida, Groupe Roussel UCLAF, Marseilles, France

R. S. Wayne and R. Lopata, American Cyanamid Co., Princeton, NJ

G. R. Winstead III and R. L. Denning, State Dept of Agriculture, Raleigh, NC

References

(1) Mount, D. L., & Miles, J. W. (1981) 181st National Meeting of the American Chemical Society, Atlanta, GA, April 2; *J. Assoc. Off. Anal. Chem.* (1982) **65**, 575–579

(2) Miles, J. W., & Mount, D. L. (1981) 25th Annual Meeting of the Collaborative International Pesticides Analytical Council, Gembloux, Belgium, June 18

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

(4) Dixon, W. J., & Massey, F. J., Jr (1957) *Introduction to Statistical Analysis*, McGraw Hill, New York, NY

ALCOHOLIC BEVERAGES

Malt Beverages and Brewing Materials: Screening and Confirmatory Methods for Nitrosamines in Beer

ANTHONY J. CUTAIA
The Stroh Brewing Co., One Stroh Dr, Detroit, MI 48226

Screening methods for nitrosamines in beer were studied by 15 collaborators. Collaborators studied 4 extraction techniques and 2 calculation procedures. All methods showed acceptable recoveries and no significant differences between methods. Based on accuracy, simplicity and speed, the Celite extraction with internal standard calculation is recommended. Fifteen collaborators also studied a distillation confirmatory method compared with a direct extraction with dichloromethane. Because the former gives an extract more suitable for mass spectrometric confirmation of nitrosamines, it is the recommended reference method. The screening and confirmatory methods have been adopted official first action.

The American Society of Brewing Chemists (ASBC) has released 2 extraction methods for nitrosamines in beer to the Associate Referee, who is also liaison between ASBC and AOAC, for recommendation for adoption as official first action. One method, recommended for screening purposes, involves Celite extraction of beer followed by dichloromethane (DCM) elution. The second method, recommended as a confirmatory procedure, is a dichloromethane extraction of a beer distillate—an operation requiring considerably more time and effort.

Normally, ASBC collaboratively tests methods of interest to the brewing industry and publishes the standardized protocol in the ASBC Journal. Assuming no untoward member reaction, these methods are published in the ASBC *Methods of Analysis* handbook. Under normal circumstances, such methods are presented to AOAC after several years of industry usage. This procedure, however, has been changed in the present instance because of the importance of the analysis and the public health implications of the class of compounds commonly known as nitrosamines.

Received September 14, 1981. Accepted December 15, 1981.

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

The recommendations of the Associate Referee were approved by the General Referee and Committee D and were adopted by the Association. See the report of the committee, March 1982 issue, for detailed recommendations.

The recommended screening method for nitrosamines in beer, using Celite 545 extraction followed by dichloromethane elution, was collaboratively tested in 1980. Fifteen collaborators participated, examining 4 extraction methods and 2 calculation procedures for each method on 4 spiked samples. The 4 methods were distillation of beer followed by dichloromethane extraction; direct dichloromethane extraction of beer; Preptube® extraction of beer followed by dichloromethane elution; and Celite 545 extraction of beer followed by dichloromethane elution. The 2 calculation procedures involved external *N*-nitrosodimethylamine (NDMA) standard addition curve, using the ratio of the NDMA peak height or area to the *N*-nitrosodipropylamine (NDPA) (internal standard) peak height or area.

For all methods, the dichloromethane eluate was concentrated and injected into a gas chromatograph equipped with either a Hall electrolytic conductivity detector modified for nitrosamine analysis or a thermal energy analyzer.

The design of the study conformed to a combined Youden unit block. Analysis of variance on all beer data, with the exception of outliers, showed no significant differences between methods ($P > 0.05$). Significant differences were noted between calculation procedures when samples contained low NDMA amounts (1–2 ppb). Combined laboratory errors expressed as coefficients of variation ranged between 7.2 and 22.8%. In general, however, the coefficients of variation were lower for the distillation and the Celite methods. The calculation procedure involving the ratio of NDMA to NDPA (internal standard) peak resulted in generally higher recoveries and values closer to theoretical than did the external standard procedure. All methods showed acceptable recoveries for the preferred calculation method with results ranging from 88 to 113% of theoretical.

Based on a combination of favorable precision (combined coefficient of variation range from 7.2 to 16.0%), reasonable accuracy (recovery range of 88.3 to 98.4%), simplicity, and speed, the Celite

0004-5756/82/6503-0584–04$01.00

method and the standard addition curve based on the ratio of NDMA to internal standard is the recommended screening method.

The recommended confirmatory method for nitrosamines in beer, using distillation followed by dichloromethane extraction of the distillate, was studied in 1979 and 1980. Fifteen collaborators participated in both studies. The distillation methods examined in both collaborative studies were identical with the exception that a 25 mL sample of beer was used in the first study and a 50 mL sample was used in the second. The 1979 study examined 2 methods applied to each of 6 spiked and 2 unspiked samples: distillation of beer followed by dichloromethane extraction of the distillate; and direct dichloromethane extraction of beer. Two calculation methods were used for the distillation method: external standard curve and external standard curve with correction for internal standard recovery. Three calculation procedures were used for the direct extraction method: the 2 mentioned above and a third procedure using a standard addition curve based on the ratio of NDMA to NDPA (internal standard).

The design of this study also conformed to a combined Youden unit block. No significant difference was seen between the 2 methods. Results based on calculation procedures using internal standards were consistently higher and more accurate. For the low, medium, and high spiked samples (about 2, 6, and 10 ppb), combined laboratory errors expressed as coefficients of variation ranged between 7.8 and 16.2%. Recoveries for methods based on internal standards ranged from 95.9 to 106.6%.

The 1980 study applied the refined calculation procedure involving standard addition based on the ratio of NDMA to NDPA to the distillation procedure. In this case, the combined laboratory errors expressed as coefficients of variation ranged between 7.9 and 14.8%. Recoveries for this method ranged from 93.9 to 105.6%.

Thus, it was seen that the distillation method, using the standard addition calculation procedure, possessed adequate precision and accuracy for recommendation as a nitrosamine detection method. However, this method also results in an extract which is more suitable for mass spectrometric confirmation of the presence of nitrosamines. Therefore, it is a preferred reference method.

Recommendation

It is recommended that the following methods for nitrosamine in beer be adopted official first action and that the official first action method for moisture in barley (**10.B01**) be adopted official final action.

N-Nitrosodimethylamine (NDMA) in Beer Gas-Liquid Chromatographic Method

Official First Action

Method I

ASBC-AOAC Method

10.C01 ***Principle***

NDMA is isolated by either adsorption on Celite or distn and is detd by GLC with either electrolytic conductivity or thermal energy analyzer with *N*-nitrosodipropylamine (NDPA) internal std.

10.C02 ***Safety Precautions***

Nitrosamines are considered potent carcinogens. Exercise extreme care in handling nitrosamines or solns of nitrosamines. Avoid skin contact. Use mech. pipetting aids for all pipetting procedures. All samples contg nitrosamines should be properly labeled as "spiked with nitrosamines" or "not for consumption," or with other adequate warning.

10.C03 ***General Precautions***

Thoroly clean all glassware used for nitrosamine analyses with Chromerge, or equiv., and thoroly rinse with H_2O and CH_2Cl_2.

Some nitrosamines degrade when exposed to UV light. Avoid prolonged exposure to fluorescent lights unless lights are covered with yellow translucent shields to filter out UV light. Alternatively, cover sample containers with foil or other suitable material to provide protection from light.

Store stds and CH_2Cl_2 exts in freezer in amber bottles or foil-covered containers.

10.C04 ***Reagents***

(**a**) *Celite 545.*—Not acid-washed (Fisher Scientific Co. No. C-212). Fire contents of each bottle 16 h at 700° before use.

(**b**) *Dichloromethane.*—CH_2Cl_2, distd in glass. (Burdick & Jackson Laboratories, Inc., or equiv.).

(**c**) *Sodium sulfate.*—Anhyd., granular.

(**d**) *Ethanol.*—Anhyd. (National Distillers and Chemical Corp., New York, NY 10016, or equiv.).

(**e**) *NDMA std soln.*—100 μg NDMA/mL alcohol (Thermo Electron Corp., 115 Second Ave, Waltham, MA 02154).

(f) *Internal std soln.*—100 ng NDPA/mL alcohol. Dil. 100 μg/mL soln (Thermo Electron Corp.) and dil. aliquot with EtOH to 100 ng/mL.

(g) *Boiling chips.*—Carborundum, small size, or equiv.; Boileezers (Fisher Scientific Co. No. B-365), or equiv.

(h) *Dry nitrogen.*—Ultra-high purity.

(i) *Aqueous ethanol.*—4% v/v, prepd with glass-distd H_2O.

(j) *Water.*—Distd in glass. H_2O processed thru deionizer may contain nitrosamines.

10.C05 ***Apparatus***

(a) *Distilling flasks.*—Round-bottom, 1 L with connecting adapter and Graham condenser set vertically.

(b) *Heating mantles.*—For 1 L flasks, with variable transformers.

(c) *Funnels.*—Fritted glass, 60 mL.

(d) *Evaporative concentrator.*—Kuderna-Danish, 250 mL capacity, 24/40 ⧩ column connection, 19/22 lower ⧩ joint. Concentrator tube size 425, 19/22 ⧩ joint, 4 mL capacity, graduated, with 19/22 ⧩ stopper. Snyder distn column, 3 sections, size 121 with 24/40 ⧩ joint (available from Kontes Glass Co., SGA Scientific, and others).

(e) *Tamping rod.*—19 mm diam. disk.

(f) *Glass wool.*—Pyrex, or equiv.

(g) *Chromatographic column.*—Glass, 28 mm id × 400 mm long with stopcock.

(h) *Gas-liquid chromatographs.*—Interfaced with thermal energy analyzer (TEA), or with Hall electrolytic conductivity detector and nitrosamine kit. The following examples of columns and conditions are suitable for nitrosamine sepn. Variations in columns and conditions are acceptable; NDMA response for 0.5 ppb beer std should be ≥5% FSD when recorder is used.

(*i*) Gas chromatograph interfaced with TEA analyzer (Model 502, Thermo Electron Corp.): 6 ft × 6 mm id glass column packed with 10% Carbowax 20M + 5% KOH on 100–120 mesh Anakrom AB; column 145°; injection port 200°; He carrier gas 35 mL/min. TEA conditions: furnace 475°; vac. with O, 1.0 torr; trap −120 to −130°.

(*ii*) Tracor Model 560/700A gas chromatograph equipped with electrolytic conductivity detector and nitrosamine detector kit: 6 ft × 6 mm id glass column packed with 15% LAC-2R-446 on 80–100 mesh Chromosorb W, acid-washed; column 140°; He carrier gas 20 mL/min; injection port 200°; Hall inlet 250°; Hall reactor 700°; H flow 50 mL/min; electrolyte, 50% v/v *n*-propanol; electrolyte flow 0.5 mL/min.

10.C06 ***Calibration Samples***

Prep. beer contg 0, 0.5, 1.0, 2.5, and 5.0 ppb (μg/L) added NDMA as follows:

Decarbonate two 12 oz bottles of beer, contg negligible NDMA content, according to **10.001,** without paper filtration. This is base beer. Prep. NDMA dil. stds from 100 μg/mL std (e), using EtOH for diln as follows:

Diln A = dil. 1.0 mL of 100 μg/mL to 10 mL
= 10 μg/mL

Diln B = dil. 5.0 mL of A to 100 mL
= 500 ng/mL

Diln C = dil. 5.0 mL of B to 10 mL
= 250 ng/mL

Diln D = dil. 2.0 mL of B to 10 mL
= 100 ng/mL

Diln E = dil. 1.0 mL of B to 10 mL
= 50 ng/mL

Add 1 mL of the following to sep. 100 mL vol. flasks: EtOH, diln E, diln D, diln C, and diln B; dil. each to vol. with previously decarbonated base beer. These samples contain 0, 0.5, 1.0, 2.5, and 5.0 ppb (μg/L) of added NDMA, resp.

10.C07 ***Celite Separation***

Carry each calibration sample thru entire procedure.

Weigh 25 ± 0.1 g decarbonated beer (**10.001,** without paper filtration) into tared 600 mL beaker. Add 1.0 mL internal std soln and 25 g Celite. Stir mixt. until uniform (ca 30 s). Mixt. will not pour but will appear light and fluffy. Place small glass wool plug in bottom of chromatgc column and cover with 20 g Na_2SO_4. Place tamping rod and powder funnel in column with end of tamping rod extending into column thru funnel. Transfer Celite mixt. to column thru funnel and tamp, a little at a time, to depth of 8–10 cm. Place K-D evaporator with 4 mL concentrator tube under column. Add 75 mL CH_2Cl_2 to beaker, swirl with spatula, and pour thru funnel before removing tamping rod. Adjust stopcock so CH_2Cl_2 flows at 1–2 mL/min into evaporator. Let column run dry (ca 35 mL CH_2Cl_2 will be recovered). Add 3 small boiling chips, fit K-D app. with distg column, and conc. to ca 4 mL in 60° H_2O bath. Let Snyder column drain, remove, and further conc. solv. to 1.0 mL under gentle stream of N at room temp. (this final concn should take ca 30 min). Inject aliquot into gas chromatograph, using either GLC/TEA or GLC/HECD conditions.

Prep. reagent blank by substituting 4% v/v EtOH in H_2O for beer, and carry thru analysis. If reagent blank shows peak for NDMA, check CH_2Cl_2 by concg 95 mL to 1 mL and chromatographing. If CH_2Cl_2 does not show NDMA peak, check other reagents. Do not use reagents showing background nitrosamines.

10.C08 ***Distillation Separation***

Carry each calibration sample thru entire procedure.

Decarbonate ca 55 mL beer by **10.001,** without paper filtration. Transfer 50.0 mL beer to 1 L r-b distn flask contg 8 g $Ba(OH)_2$ and Boileezers. Add 1.0 mL internal std soln. Distill slowly (variable transformer setting 60%), collecting ca 48 mL in ice-cooled 250 mL separator. Add 0.4 g Na_2CO_3. Ext 4 times with 20 mL portions of CH_2Cl_2, shaking each for 1 min. Pool exts in second 250 mL separator. Pass ext thru 30 g Na_2SO_4 (held in 60 mL fritted glass funnel prewetted with CH_2Cl_2) into 250 mL K-D evaporator with 4 mL concentrator tube attached. Wash Na_2SO_4 with 15 mL CH_2Cl_2 and add wash to evaporator flask. Add one Boileezer and Snyder column and carefully conc. to 4 mL in 60° H_2O bath. Remove Snyder column and further conc. to 1.0 mL under gentle stream of N at room temp. (this final concn should take ca 30 min). Inject aliquot into gas chromatograph, using either GLC/TEA or GLC/HECD conditions.

Prep. reagent blank as in **10.C07.**

10.C09 ***Calculations***

Measure peak ht (or area) of NDMA and NDPA (internal std) peaks on chromatograms and det. ratio

$$R = \frac{\text{peak ht (or area) NDMA}}{\text{peak ht (or area) NDPA}}$$

Subtract R for 0 ppb calibration sample from R values obtained for other calibration samples. Prep. std curve by plotting ppb added NDMA vs R values for each NDMA calibration level (after subtraction of R for 0 ppb).

Calc. slope and intercept of regression line, using method of least squares where X = NDMA (μg/L) and $Y = R$ value.

Calcn of unknowns: Measure peak ht (or area) for NDMA and NDPA peaks and calc. R as above. Det. μg/L in beer by calcn using regression equation and solving for X as follows:

$$\mu g/L = \frac{R - \text{intercept}}{\text{slope}}$$

Report results to one decimal place.

PRESERVATIVES AND ARTIFICIAL SWEETENERS

Gas Chromatographic Determination of Sorbitol, Mannitol, and Xylitol in Chewing Gum and Sorbitol in Mints

DANIEL H. DANIELS, CHARLES R. WARNER, and THOMAS FAZIO
Food and Drug Administration, Division of Chemistry and Physics, Washington, DC 20204

A method has been developed for determination of sorbitol, mannitol, and xylitol in chewing gum and sorbitol in mints. Chewing gum is partitioned between methylene chloride and water; the mint is simply dissolved in water. The aqueous extract is dried and the residue is derivatized with pyridine-acetic anhydride to form the corresponding peracetates. The derivatives are quantitated by gas chromatography using a 9 ft × 2 mm column packed with 10% Silar 10C on Chromosorb W/AW. Average recoveries of these sugar alcohols ranged from 96 to 102%.

In recent years, the need to quantitate D-mannitol (mannitol), D-glucitol (sorbitol), and xylitol in sugarfree mints and chewing gum has been recognized. Sorbitol is a sweetener that is generally recognized as safe for use in foods (1); nevertheless, sorbitol content in food may not exceed certain levels that are based on current good manufacturing practice. Sorbitol is presently permitted at maximum levels of 98 and 99% in soft and hard candy, respectively, and 75% in chewing gum (2). Maximum allowable levels of mannitol are 98% in pressed mints and 31% in chewing gum (3). A preliminary survey in our laboratory indicated that sugarfree mints contain sorbitol as the exclusive sweetener at the highest permissible levels, whereas sugarfree chewing gums contain 50 to 75% sorbitol and approximately 5% mannitol. Xylitol is not currently used in the United States; xylitol is found, however, in Canadian samples of chewing gum. This paper presents a simple, rapid gas chromatographic (GC) method for determination of these 3 substances, which are known collectively as sugar alcohols, in chewing gum and mints.

Several researchers have determined sugar alcohols by gas and liquid chromatography (LC). Solutions have been analyzed by GC after conversion of the sugar alcohols to the corresponding fully acetylated esters (4–6) or trimethylsilyl ethers (7, 8). Sondack (9) and Brooks and Maclean (10) utilized the *n*-butylboronic esters for the GC determinative step. Samarco (11) partitioned the sugarless chewing gum between toluene and water. The aqueous solution was analyzed by LC. Our objective was to develop a method based on a single GC separation, which would permit quantitation of the sugar alcohols commonly found in food.

In our work we partitioned the chewing gum samples, using a methylene chloride–water system, and extracted sorbitol from the mints with water. After removal of water from an aliquot, we then formed peracetate derivatives, using pyridine–acetic anhydride; the derivatives were determined using a 9 ft × 1/4 in. od (2 mm id) column containing 10% Silar 10C on Chromosorb W/AW.

Experimental

Apparatus

(a) *Gas chromatograph.*—Varian Model 3700 (Varian Associates, Inc., Instrument Group, Palo Alto, CA 94303), or equivalent, equipped with flame ionization detector (FID). Column: 9 ft × 1/4 in. od (2 mm id) glass containing 10% Silar 10C on 60–80 mesh Chromosorb W/AW. Operating conditions: column temperature, 200°C; injector temperature, 250°C; detector temperature, 330°C; nitrogen flow rate, 20 mL/min.

(b) *Syringe.*—10 μL microsyringe (Hamilton Co., Reno, NV 89510, or equivalent).

(c) *Boileezer boiling chips.*—(Fisher Scientific Co., Pittsburgh, PA 15219) ground to a fine powder. Isolate fraction which passes through 20 mesh screen but is retained by 40 mesh screen.

(d) *Oxford sampler.*—50–200 μL range and 200–1000 μL range plus disposable tips (Fisher Scientific Co.)

(e) *Vials.*—3 mL vials with Teflon-lined screw caps (PGC Scientifics Corp., Gaithersburg, MD 20877, or equivalent).

(f) *Heater-mixer.*—Buchler Vortex-Evaporator (Buchler Instruments, Inc., Fort Lee, NJ 07024), or equivalent, equipped to hold 3 mL vials. This

Received October 8, 1981. Accepted November 18, 1981.
This paper was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

0004-5756/82/6503-0588-04$01.00

instrument must be capable of simultaneously heating vial and subjecting it to vortex swirling at 3–5 excursions/s.

(g) *Centrifuge.*—Sorvall RC-3 (DuPont Instruments-Sorvall Biomedical Div., Newton, CT 06470), or equivalent, equipped to hold 250 mL plastic centrifuge bottles and operate at 3000 rpm.

(h) *Centrifuge bottles.*—250 mL plastic (Fisher Scientific Co., or equivalent).

(i) *Vacuum oven.*—National Appliance Co., Portland, OR 97204.

Reagents

(a) *Methylene chloride.*—Distilled in glass (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442).

(b) *Acetic anhydride–pyridine (1 + 1).*—In 50 mL graduated cylinder add 25 mL acetic anhydride (Fisher Scientific Co., or equivalent) and 25 mL pyridine (Fisher Scientific Co., or equivalent).

(c) *L-Inositol.*—ICN Nutritional Biochemicals, Cleveland, OH 44128.

(d) *Sorbitol (97%), mannitol, and xylitol.*—Aldrich Chemical Co., Inc., Milwaukee, WI 53233.

(e) *Sorbitol hexaacetate, xylitol pentaacetate, and mannitol hexaacetate.*—Chemical Standard Quality (Supelco, Inc., Bellefonte, PA 16823).

Preparation of Standard Curve

Chewing gum.—Quantitatively transfer to each of three 25 mL volumetric flasks 0.5X, X, and 1.5X mg of each sugar alcohol, where X is quantity in mg expected in analytical sample. Add 20 mg L-inositol as internal standard to each flask. Dilute to volume with water. Continue as described in Determination.

Mints.—Quantitatively transfer to each of three 100 mL volumetric flasks 0.5X, X, and 1.5X mg of each sugar alcohol except xylitol, where X is quantity in mg expected in analytical sample. Add 50 mg xylitol to each flask. Dilute to volume with water. Continue as described in Determination.

Plot relative response, R, vs mg sugar alcohol of interest transferred to flask where R = peak height of sugar alcohol of interest/peak height of internal standard.

Sample Preparation

Chewing gum.—Thoroughly chop and dice 1 stick chewing gum with single edge razor blade. Transfer 1.0 g finely divided chewing gum to 250 mL plastic centrifuge bottle. Add 50 mL methylene chloride and 25 mL water. Add 20 mg L-inositol as internal standard. Shake until chewing gum is thoroughly dispersed. Take known quantity of aqueous phase for analysis as indicated under Determination. Centrifuge 20 min at 3000 rpm to separate aqueous and organic layers. Proceed as directed under Determination.

Mints.—Break up mints by any convenient process. Transfer an accurately weighed quantity of pieces equivalent to ca 1 g to 100 mL volumetric flask. Add 0.5 g xylitol as internal standard. Add 75 mL water. Shake until material is thoroughly dispersed. (Excipients will still be visible in solution; however, sugar alcohol will be dissolved.) Dilute to volume with water. Also prepare blank sample (without xylitol internal standard) for GC analysis to ensure that area corresponding to retention time of xylitol is free from interferences. Continue as described in Determination.

Determination

Using 50–200 μL Oxford sampler system with disposable tips, transfer known aliquot (based on estimated sugar alcohol content of sample) of aqueous phase of sample to 3 mL vial and add 1 mg finely ground boiling chips. Evaporate sample to dryness in 50°C oven under vacuum of 10 in. Hg. Cool to room temperature and add 400 μL acetic anhydride–pyridine reagent, using Oxford sampler system with disposable tips. Mix and heat 1 h at 80°C in Buchler Vortex-Evaporator. Inject 0.5–3 μL solution into gas chromatograph. Attenuation of GC system must be accomplished by automatic or manual means during chromatographic analysis to keep all peaks on scale. (After more than 100 injections the column showed no signs of deterioration.)

Calculations

Calculate relative response, R, of each sugar alcohol of interest for sample, using equation described for standard curve. From standard curve determine quantity (mg) of sugar alcohol present in sample. Calculate mg sugar alcohol/g sample. Calculate % (w/w) sugar alcohol in sample according to the following equation:

$$\% \text{ (w/w)} = (\text{mg sugar alcohol/g sample}) \times (100/1000)$$

Recovery Studies

Recovery studies were carried out by spiking 1 g chewing gum which was free of sorbitol, mannitol, and xylitol but carried a label declaration: sugar, gum base, corn syrup, natural and

Table 1. Recovery of sugar alcohols added to chewing gum

Sugar alcohol	Level, % (w/w)	Rec.,[a] %	CV, %
Mannitol	0.5	99.8	0.92
	1	100.8	0.65
	5	98.8	2.28
Sorbitol	5	100.8	0.71
	10	101.3	1.12
	33	97.9	2.21
Xylitol	5	101.5	1.32
	10	99.1	1.90
	33	96.3	1.33

[a] Each recovery value is the average of 8 determinations.

artificial flavoring, and artificial colors. The samples were spiked at each of 3 levels for xylitol, mannitol, and sorbitol as indicated in Table 1. For example, the lowest spiking level, 0.5% mannitol, 5% sorbitol, and 5% xylitol, was achieved by adding 4 mL of an aqueous solution containing 1.25 mg mannitol/mL, 12.5 mg sorbitol/mL, 12.5 mg xylitol/mL, and 5 mg L-inositol/mL (internal standard). To ensure an adequate challenge for the method, the bottles were shaken until the gum base was thoroughly dispersed in the solvents. The recovery studies were completed as described under Determination.

Results and Discussion

Figure 1 illustrates the GC separation of a standard mixture of the peracetate derivatives of xylitol, mannitol, and sorbitol. As can be seen, the sugar alcohol derivatives are well resolved. No interferences were encountered with any of the sucrose-containing chewing gum samples selected for the recovery studies. These samples contained sugar, gum, and corn syrup along with spearmint and cinnamon flavorings.

The results given in Table 1 demonstrate the accuracy and precision with which this method can be used to determine sugar alcohols in chewing gum. To establish the identities of the peracetates formed in the reaction mixture, the retention times were compared to those of the known peracetates. Each of the products had a retention time identical to that of the corresponding reference standard peracetate. Formation of the peracetate derivative was 98–113% efficient as indicated by FID response relative to equimolar amounts of the reference standard peracetates.

Table 2 shows the amounts of mannitol, sorbitol, and xylitol in commercial sugarfree chewing gums. In each case the mannitol content is below the legally permitted level of 31% (3). The Canadian sample of Brand A is the only chewing gum sample containing xylitol, which

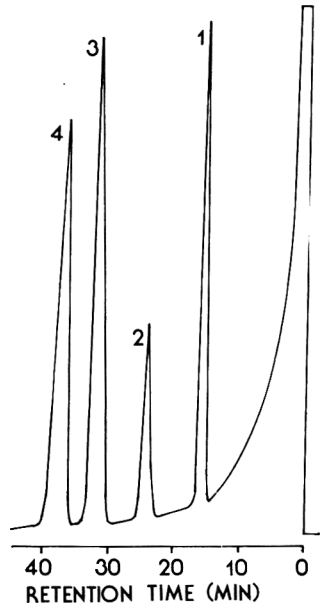


Figure 1. GC separation of sugar peracetate standards (μg, as sugar alcohol on-column): 1, xylitol (0.16); 2, mannitol (0.16); 3, sorbitol (0.32); 4, L-inositol (0.32).

Table 2. Sugar alcohols found in commercial samples of sugarfree chewing gums

Gum sample	Sugar alcohol	Found[a] mg/g	Found[a] % (w/w)
Brand A			
Spearmint	mannitol	18	1.8
	sorbitol	717	71.7
Peppermint	mannitol	16	1.6
	sorbitol	711	71.1
Peppermint (Canadian)	mannitol	98	9.8
	sorbitol	504	50.4
	xylitol	137	13.7
Brand B			
Bubble gum	mannitol	94	9.4
	sorbitol	593	59.3
Brand C			
Fruit flavor	mannitol	33	3.3
	sorbitol	691	69.1
Cinnamon	mannitol	32	3.2
	sorbitol	701	70.1

[a] Sorbitol results are corrected for 97% purity of reference standard.

Table 3. Sorbitol found in commercial samples of sugarfree mints

Mint sample	Sorbitol found[a] mg/g	% (w/w)
Brand A		
Peppermint	970[b]	97.0
Fruit	980	98.0
Wintergreen	970	97.0
Brand B		
Wintergreen	1000	100.0
Cinnamon	1000	100.0
Brand C		
Spearmint	975	97.5

[a] Results corrected for 97% purity of sorbitol reference standard. Values are based on 2 determinations.
[b] Based on 8 determinations; CV, % = 0.861.

is not used in chewing gum produced in the United States. Table 3 gives the results obtained with sugarfree mints. The composition of the mints proved to be mostly sorbitol with added excipients and flavoring agents.

The methodology described in this report gives accurate and precise assays of sugar alcohols in chewing gum and mints. The calibration curves of response vs quantity of sugar alcohol were linear with a coefficient of correlation >0.99. Recovery studies (Table 1) were designed to test the methodology at or below the levels commonly found in chewing gum.

While developing a convenient and reliable method, we evaluated the essential elements of a number of other procedures. The *n*-butylboronate esters of mannitol and sorbitol produced skewed peaks with the columns we used. The trimethylsilyl ethers could not be formed reproducibly in the presence of moisture. In many cases derivative/column combinations did not give complete resolution of the sugar alcohols and the internal standard. The method described above produces excellent results with mints and chewing gum.

In summary, a method has been described which permits the determination of sugar alcohols in chewing gum and mints. The method utilizes peracetate derivative formation of the sugar alcohol followed by GC analysis. We found this method rapid and easy to use.

REFERENCES

(1) *Code of Federal Regulations* (1981) Title 21, U.S. Government Printing Office, Washington, DC, sec. **182.90**
(2) *Code of Federal Regulations* (1981) Title 21, U.S. Government Printing Office, Washington, DC, sec. **184.1835**
(3) *Code of Federal Regulations* (1981) Title 21, U.S. Government Printing Office, Washington, DC, sec. **180.25**
(4) Manius, G., Mahn, R. P., Venturella, V. S., & Senkowski, B. Z. (1972) *J. Pharm. Sci.* **61,** 1831–1835
(5) Whyte, J. N. C. (1973) *J. Chromatogr.* **87,** 163–168
(6) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **20.151-20.156**
(7) Freeman, E. H., Stephen, A. M., & Van Der Bijl, P. (1972) *J. Chromatogr.* **73,** 29–33
(8) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **22.080-22.082**
(9) Sondack, D. L. (1975) *J. Pharm. Sci.* **64,** 128–129
(10) Brooks, C. J. W., & Maclean, I. (1971) *J. Chromatogr. Sci.* **9,** 18–24
(11) Samarco, E. C. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1318–1320

BIOCHEMICAL METHODS

Procedures for Measuring Accuracy and Sensitivity of Immunochemical Pregnancy Test Kits

LILLIAN J. GILL
Food and Drug Administration, 900 Madison Ave, Baltimore, MD 21201

Analytical procedures were used to measure the accuracy and sensitivity of immunochemical pregnancy test kits. Performances of all currently marketed hemagglutination inhibition, latex agglutination inhibition, and direct latex agglutination pregnancy kits were evaluated.

Human chorionic gonadotropin (HCG) is a glycoprotein that is secreted during pregnancy from the time of the formation of the syncytiotrophoblast throughout the growth of the placenta. The protein is secreted into the blood and appears in the urine after glomerular filtration, reaching its highest level during the first trimester (1). Qualitative analysis for HCG is used primarily to detect pregnancy, whereas quantitative analyses can indicate certain pathological conditions and monitor the progress of therapy in these conditions.

Four immunochemical methods are currently available for measuring HCG: latex agglutination inhibition (LAI), hemagglutination inhibition (HAI), direct latex agglutination (DLA), and radioimmunoassay (RIA). The LAI and HAI procedures are based on antigen–antibody reactions in which latex particles (for the LAI method) or erythrocytes (for the HAI method) are sensitized with HCG antigen and used as the test indicator. The DLA procedure, also based on an antigen-antibody reaction, uses latex particles coated with anti-HCG. Biological tests (2) as well as the RIA and the more modern radioreceptor assays (RRA) (3) are also used in pregnancy testing. However, our study evaluated only the immunochemical agglutination reaction tests.

Recent studies have compared immunochemical procedures with one another and with other diagnostic techniques to determine their quality and reliability. In comparing immunochemical methods to RRA, Roy et al. (4) found that although HAI was less sensitive than RRA in detecting early pregnancies, its positive and negative results were just as accurate. They also showed that the clinical sensitivity of the LAI was poorer than the HAI and RRA. In a study of 5 commercial immunochemical pregnancy kits, Horwitz et al. (5) concluded that the HAI kits were more accurate and sensitive than the LAI or DLA kits.

In August 1977, the Food and Drug Administration (FDA) began analytical evaluation of the performance of various immunochemical pregnancy test kits used to detect HCG. The testing included validation of consumer complaints, evaluation of the performance of kits which had been submitted by the manufacturer for release approval, and development of laboratory protocols for possible future compliance programs. The need for a standardized, transferable analytical method for evaluating kit performance was reinforced by increased over-the-counter sales of these products. The protocols developed for testing the performance of these kits are presented here along with data on the tests of manufacturers' claims for 206 assorted lots of these products.

Experimental

Principle

Antiserum (antibody) is produced in rabbits that have been injected with HCG. For the HAI reaction, erythrocytes used as an indicator are coated with HCG (antigen). When the anti-HCG serum reacts with the HCG-coated indicator particles, agglutination (precipitation) occurs. However, when urine from a pregnant woman, which contains HCG, is mixed with the antiserum, the antiserum is neutralized by antigen in the urine, and agglutination of erythrocytes is inhibited. The erythrocytes then settle in a doughnut pattern at the bottom of the round-bottom test vial. Urine from a nonpregnant female, which contains no HCG and therefore does not inhibit agglutination, reacts with the erythrocytes to form a brownish yellow homogeneous solution.

The LAI reaction is also based on this antigen-antibody principle, except that latex particles

Received April 15, 1981 Accepted November 3, 1981

0004-5756/82/6503-0592—06$01.00

coated with HCG are used as the indicator instead of erythrocytes. When antiserum is mixed with a suspension of these coated latex particles, agglutination occurs, indicating a negative reaction. When urine from a pregnant woman is mixed with the antiserum, the antiserum is neutralized by the HCG in the urine, and agglutination with the latex particles is inhibited; thus, no agglutination occurs and the result is positive. This kit has both slide and tube varieties.

Unlike HAI and LAI, the DLA immunochemical principle involves fixation of the antibody (as opposed to antigen) to the latex particles. In the presence of the HCG antigen (as in pregnancy) these latex particles agglutinate. In DLA testing, agglutination indicates the presence of HCG (pregnancy) and no agglutination indicates the absence of HCG in the sample (no pregnancy).

Apparatus

For HAI use **a**-**c**; for LAI use **a**, **b**, and **d**; for DLA use **a**, **b**, and **d**.

(**a**) *Filter paper.*—Whatman No. 4 or equivalent, or centrifuge (IEC, clinical, or equivalent) capable of 1000 × g.

(**b**) *Precalibrated droppers (supplied in kit) or pipets.*—Capable of delivering 100 μL (for each urine specimen) and 400 μL (for buffer). Pipets can be fixed volume (e.g., Eppendorf) or graduated.

(**c**) *Mirrored rack.*—Test rack must hold test vials absolutely vertical over angled mirror (ca 30°) to facilitate reading of results without disturbing rack or tubes.

(**d**) *Glass or paper slide.*—With circles outlined on background for detection of agglutination (precipitation).

Reagents

(**a**) *Antiserum, antigen.*—Both supplied in kit. Materials may be premeasured liquids or freeze-dried solids.

(**b**) *Buffer.*—Supplied in kit. Prepare according to manufacturer's instructions.

(**c**) *Primary stock solution.*—International reference standard for HCG, 10 IU HCG/mL aliquots (World Health Organization, obtained through FDA, Bureau of Medical Devices, Silver Spring, MD 20910). An international unit (IU) is defined as the biological activity of the hormone in relation to an existing international standard. In this study an IU is based on the activity for HCG in 1.279 μg of the second international standard.

(**d**) *Positive and negative controls.*—Filter or centrifuge urine from clinically confirmed pregnant and nonpregnant human females. If controls are not supplied by manufacturer, use negative pooled urine or physiological saline. Analyze and confirm individual negative urines from nonpregnant females before pooling. Prepare positive controls by adding HCG to urine or saline.

(**e**) *Physiological saline.*—8.5 g NaCl in 1 L distilled water. Caution: Lyophilized reagents are hygroscopic. Do not expose to atmosphere until just before use. Do not use reagents which are shrunken or hydrated. Do not freeze kits. Reagents are damaged by extremes of temperature. Refrigerate kits at 2–8°C.

Accuracy Determination

Prepare a panel of urines consisting of 10 known positives and 10 known negatives. The urines must be female patient specimens which have been assayed for HCG content; they must not contain protein or other substances which could interfere with this procedure. Vary specimens in panels so that positives and negatives are from as many different sources as possible. Include some positive specimens from patients in their first month of pregnancy. Filter or centrifuge urines 5 min at ca 1000 × g. Use only filtrate or supernate.

Sensitivity Determination

Prepare a series of dilutions of the reference standard using known pooled negative urines (collected from nonpregnant females and confirmed negative before pooling) as diluent within the sensitivity range stated on kit. Prepare dilutions fresh daily and include at least 2 concentration levels above and below the declared sensitivity factor. If declared sensitivity of kit is 1, ≤1–2, or ≥2, prepare HCG concentrations in increments of 0.1, 0.2, and 0.3 IU/mL, respectively.

Test Procedures

Perform test on urine panel and prepared reference standard solutions exactly as stated in instructions included with kit sample (information insert). Use apparatus and reagents supplied with kit. If sample is received in bulk form or test instructions are not supplied, perform test on urine panel and on prepared reference standard solutions.

HAI.—Add 0.1 mL urine or appropriate reference standard dilution to round-bottom vial or test tube containing manufacturer's premeasured

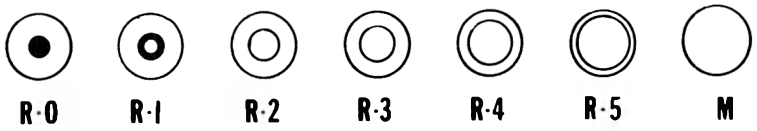


Figure 1. Interpretation of results for HAI test. R-0 = solid small button cells (positive); R-1 = thick ring of cells with small clear area in center of tube (positive); R-2 = thick ring of cells with periphery of ring at half or less than half the radius from center of tube (positive); R-3 = thinner but definite ring at half the radius (positive); R-4 = thin but definite ring closer to periphery of tube (inconclusive, repeat); R-5 = very thin or indefinite ring at the periphery (inconclusive, repeat); M = smooth mat of cells with no ring (negative).

amounts of anti-HCG serum and HCG-coated erythrocytes for lyophylized products, or anti-HCG serum from calibrated droppers for liquid reagent kits. Swirl gently to mix. For freeze-dried reagents, promptly add 0.4 mL buffer to each test vial. For liquid reagents, add 2 drops of well mixed HCG-coated erythrocytes.

Rotate rack in circular motion ca 120 rpm. Let rack stand undisturbed at ambient room temperature; avoid vibrations and extremes of heat. Read results after 2 h. Note: Positive results may be read as early as 1 h after test has begun; however, 2 h should expire before negative result is considered valid. No agglutination, which is a positive reaction, is expressed by a distinct red-brown ring at the bottom of the tube (Figure 1). Negative samples show agglutination as a yellow-brown homogeneous solution. Irregular, broken, or ill-defined rings are considered inconclusive and may be due to inadvertent disturbances of the rack, unclean glassware, or presence of a foreign body. These samples should be retested.

LAI (slides).—At 90° angle to glass or paper slide and in center of circle, place 1 drop (50 μL) of anti-HCG serum. Add 1 drop (50 μL) of urine or reference standard dilution to anti-HCG serum. Use provided dropper or micropipet. Mix with applicator sticks until mixture is homogeneous over entire area of circle. Slowly rotate slide in circular motion for 30 s. Thoroughly mix latex suspension by gentle inversion and add 1 drop (50 μL) of suspension to urine-antiserum mixture. Gently rotate slide in circular motion for 2 min. Read results.

LAI (tubes).—Into 10 × 75 mm test tube containing 2 mL antiserum reagent (supplied by manufacturer) add 1000 μL urine or reference standard dilution. Add 2 drops of thoroughly mixed latex suspension with supplied dropper. Cap tube with its fitted stopper and invert 4 times to ensure proper mixing. Place tube in a 37 ± 2°C water bath or heating block. Read results after 90 min of incubation. Lack of agglutination, which is a positive reaction, appears as a smooth milky suspension on slides after 2 min and after 90 min in tubes. Negative reactions may display any degree of precipitation (+, ++, or +++).

DLA.—Thoroughly mix latex suspension. At 90° angle to glass or paper slide and in center of circle, place 1 drop (50 μL) of latex suspension. Add 1 drop (50 μL) of urine or appropriate reference standard dilution. Stir with applicator sticks until mixture is homogeneous over entire area of circle. Slowly rotate slide in circular motion for 1 min. Read results. Positive reactions show various degrees of precipitation (+, ++, or +++) (Figure 2). A negative reaction shows no agglutination and is evidenced by a smooth milky solution.

Results and Discussion

Table 1 presents a list of pregnancy kits submitted by 6 different manufacturers and tested in our laboratory from January 1978 to April 1979. The products included samples of every immunochemical pregnancy test kit marketed as of December 30, 1978. The samples were received in the form intended for clinical or hospital laboratory use: Some kits were ready to use; others were received in bulk form without labeling, packaging, or apparatus. The analyz-

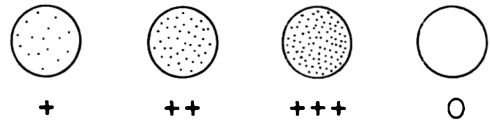


Figure 2. Interpretation of results for LAI and DLA tests. LAI: 0 = total inhibition, no agglutination, smooth milky appearance (positive); + = tiny clumps or fine precipitate at end of time period (negative); ++ = small clumps throughout (negative); +++ = maximum agglutination (negative). DLA: 0 = no agglutination (negative); + = tiny clumps before or at end of time period (positive); ++ = small clumps throughout (positive); +++ = maximum agglutination (positive).

Table 1. Immunochemical pregnancy test kits on market as of December 30, 1978

Product	Manufacturer	Type of test	Type of reagent (antigen–ant body)	Stated reaction time (min)	Range of stated sensitivities (IU/mL)
Placentex	Hoffmann-LaRoche	LAI tube	liquid	90	1.25
Pregnosis	Hoffmann-LaRoche	LAI slide	liquid	2	2.0
Gest-State	Lederle/Fisher	LAI slide	liquid	2	2–4
Neogestic[a]	Organon	LAI slide	dried on slide	2	1–2
Planosec[a]	Organon	LAI slide	dried or slide	2	1–2
Dri-Dot	Organon	LAI slide	dried or slide	2	1–2
Pregnosticon Slide	Organon	LAI slide	liquid	2	2
Gravindex	Ortho	LAI slide	liquid	2	3.5
UCG-Slide	Princeton	LAI slide	liquid	2	2.0
Pregna-B	International Diagnostics	LAI slide	liquid	2	2
D.A.P. Test	Princeton	DLA slide	liquid	2	2–1000
Pregnosticon Accuspheres	Organon	HAI tube	lyophilized	120	0.65–0.7
Neocept	Organon	HAI tube	lyophilized	120	0.2
UCG-Tube	Princeton	HAI tube	liquid	120	0.5
UCG-Lyphotest	Princeton	HAI tube	lyophilized	120	0.7
UCG-Titration	Princeton	HAI tube	liquid	120	1.0
UCG-Quiktube	Princeton	HAI tube	liquid	120	1.0
Pregna-B	International Diagnostics	HAI tube	lyophilized	120	0.7

[a] Names on exported Dri-Dot kits.

ing capacity of the products ranged from 10 to 300 tests. Of the kits submitted, 56% were LAI, 39% were HAI, and 5% were DLA. Of the 206 lots tested, 42% were LAI, 55% were HAI, and 3% were DLA.

Results of the qualitative accuracy tests are given in Table 2. A lot-by-lot breakdown of defective samples is shown in Table 3. Each kit was tested on a panel of 20 human urine specimens consisting of 10 known positives and 10 known negatives. These clinically confirmed specimens were randomly selected from more than 4000 frozen specimens from approximately 200 different patients. The urines were frozen in 3–5 mL portions to eliminate the possibility of bacterial growth caused by refreezing or resampling from the same container over a period of days. A brief medical history accompanied each specimen.

To minimize personal bias in interpreting re-

Table 2. Accuracy of LAI, DLA, and HAI kits

Test	Product	No. of lots tested[a]	No. of tests performed	Positives		Negatives		False positives	False negatives	Inconclusives
				No.	% Correct	No.	% Correct			
LAI	Placentex	18	360	180	100	180	100	0	0	0
	Pregnosis	6	120	58	96.7	56	93.3	4	2	0
	Gest-State	3	60	30	100	30	100	0	0	0
	Dri-Dot	11	220	110	100	109	99.1	1	0	0
	Pregnosticon Slide	11	220	104	94.5	101	91.8	7	6	2
	Gravindex	13	260	130	100	130	100	0	0	0
	UCG-Slide	22	440	219	99.5	216	98.2	3	1	1
	Pregna-B Slide	3	60	30	100	30	100	0	0	0
Total LAI		87	1740	861	99.0	852	97.9	15	9	3
DLA	D.A.P. Test	6	120	60	100	60	100	0	0	0
HAI	Accuspheres	33	660	330	100	330	100	0	0	0
	Neocept	7	140	70	100	70	100	0	0	0
	UCG-Tube	23	460	230	100	230	100	0	0	0
	UCG-Lyphotest	23	460	230	100	230	100	0	0	0
	UCG-Quiktube	10	200	100	100	100	100	0	0	0
	Pregna-B Tube	2	40	20	100	20	100	0	0	0
Total HAI		98	1960	980	100	980	100	0	0	0

[a] Each sample was tested on 10 known positive and 10 known negative urine specimens.

Table 3. Accuracy study of defective LAI kits tested by lot

Product	No. of lots tested[a]	No. of lots defective	Lot No.	Specimens correct No.	Specimens correct %	False positive No.	False positive %	False negative No.	False negative %	Inconclusive No.	Inconclusive %
Pregnosis	6	1		14	70	4	20	2	10	0	0
Dri-Dot	11	1		19	95	1	5	0	0	0	0
Pregnosticon Slide	11	2	A	10	50	4	20	4	20	2	10
			B	15	75	3	15	2	10	0	0
UCG-Slide	22	2	A	18	90	1	5	0	0	1	5
			B	17	85	2	10	1	5	0	0

[a] Each lot was tested on 10 known positive and 10 known negative urine specimens.

sults, the known urines were set up as a "blind" panel. The numbered specimens were known at time of selection to ensure an equal number of positives and negatives, then rearranged so that their identities remained unknown during the testing. These 20 urines were tested as regular unknown specimens; results were recorded and compared with clinical results.

False positives were defined as those which gave positive results on confirmed negative urines; those which gave negative results on confirmed positive urines were defined as negative. The guides used to interpret the results for each of the 3 techniques are shown in Figures 1 and 2. Because the UCG-Titration kit is manufactured exclusively to quantitate HCG, the 15 lots received for evaluation were included only in the sensitivity study.

None of the 98 HAI kits tested gave false positive or false negative results. The LAI kits gave 24 false and 3 inconclusive results. The data showed that the HAI kits were more accurate than the LAI kits. The data in Table 2 seemed to favor DLA over LAI kits; however, the DLA kits have been reported to display the prozone phenomenon, i.e., lack of agglutination in the presence of excessive amounts of antigen or antibody (1, 5).

Nine products were tested further for sensitivity of HCG detection. Although selection was limited by the amount of sample available, a representative number of each of the 3 types of immunochemical kits was included from a variety of manufacturers. Two additional lots each of Pregnosis and DAP kits not evaluated in the accuracy testing were included in the sensitivity study (Table 4).

Each kit was subjected to a urine dilution panel, ranging from 0.6 to 2.75 IU HCG/mL. If the declared sensitivity was between 1.0 and 2.5 IU/mL, urine standards in increments of 0.2–0.25 IU/mL were used. If the declared sensitivity was ≤1.0 IU/mL, urine standards in increments of 0.1 IU/mL were used. An example of the latter scheme is shown in Table 5.

HAI kits with a declared sensitivity of 0.65 IU/mL gave a number of faint patterns. These questionable reactions were not included in calculating the mean sensitivity values. The mean level of HCG detected by HAI kits was

Table 4. HCG sensitivity mean and range of LAI, DLA, and HAI kits

Type of test	Product	No. of lots tested	Declared sensitivity (IU/mL)	Av. sensitivity (IU/mL)	Sensitivity range (IU/mL)
LAI	Pregnosticon Slide	11	2.0	1.64	1.2–2.25
	UCG-Slide	10	2.0	1.92	1.8–2.2
	Placentex	10	1.25	0.96	0.8–1.2
	Pregnosis	8	2.0	2.08	1.8–2.25
Mean			1.8	1.65	1.4–1.98
DLA	D.A.P. Test	8	2–1000	1.98	1.8–2.2
HAI	UCG-Titration	15	1.0	0.93	0.7–1.25
	UCG-Lyphotest	10	0.7	0.65	0.5–0.8
	UCG-Quiktube	10	1.0	0.93	0.8–1.0
	Accuspheres	10	0.7	0.56	0.5–0.6
Mean			0.85	0.77	0.63–0.91

Table 5. Dilution scheme for HCG sensitivity test for kits with declared sensitivity of ≤1.0 IU/mL[a]

Concn (IU HCG/mL)	Intermediate std (mL)	Pooled negative urine diluent (mL)
1.0	0.50	1.50
0.9	0.45	1.55
0.8	0.40	1.60
0.7	0.35	1.65
0.6	0.30	1.70
0.5	0.25	1.75
0.4	0.20	1.80
0.3	0.15	1.85
0.2	0.10	1.90
0.1	0.05	1.95

[a] Intermediate standard: 2 mL of 10 IU HCG/mL WHO reference std + 3 mL pooled urine = 5 mL of 4 IU HCG/mL.

below that obtained with LAI and DLA kits (Table 4). Thus, the HAI detects pregnancy at an earlier stage. Average sensitivity of all kits tested was greater than or equal to that claimed by the manufacturer. It was concluded that the HAI kits provided a more accurate and sensitive measurement of HCG in urine and easier interpretation of test results than the LAI and DLA kits.

The following points should be noted in the evaluation of pregnancy kit performance: (a) Denaturation of the antisera may enhance the sensitivity and give an increase of false positive reactions (6). Therefore, both positive and negative controls must be used to provide a check on reagent quality. (b) Low concentrations of HCG standard in dilute solutions, i.e., <10 IU/mL were unstable because of adsorption of HCG on glass surfaces. Thus, dilutions must be prepared fresh daily. (c) False positive results may be caused by HCG secreted in pathological conditions, e.g., hydatidiform moles, choriocarcinoma, and certain tumors (7). Low HCG titers, as in ectopic pregnancy (7), may give false negatives. Large amounts of protein in urine can neutralize HCG antiserum, producing false positives (8). Drugs, such as phenothiazines and methadone, can also give erroneous results (9). False positive urine from menopausal women (8) may be checked by retesting a 1:2 dilution of the specimen. (d) Data from repeat analyses could be misleading. Improper packaging, absorption of moisture, and nonuniformity in mixing could cause a reagent to give incorrect results on the first analysis and correct results on subsequent re-analysis; thus an analyst may wrongly conclude that the product was functioning properly. All false and inconclusive results must be reported. (e) Reagents should be visually inspected before use to detect bad reagents, as evidenced, for example, by clumping of the latex or erythrocyte suspensions. In the HAI test, jagged rings which appear to contain particles may indicate a problem with the lyophilization process. Faintly positive rings may be due to an inadequate number of red cells in the suspension.

Acknowledgment

The author thanks Willard Stewart, FDA, Bureau of Medical Devices, and William M. Ment, FDA, Baltimore, MD, for their assistance, suggestions, and encouragement.

References

(1) Mishell, D. R., Jr., Wide, L., & Gemzell, C. A. (1963) *J. Clin. Endocrinol. Metab.* **23**, 125–131
(2) Hobson, B. M. (1966) *J. Reprod. Fertil.* **12**, 33–48
(3) Landesman, R., & Saxena, B. B. (1976) *Fertil. Steril.* **27**, 357–368
(4) Roy, S., Klein, T., Scott, J. Z., Kletzky, O. A., & Mishell, D. R., Jr (1977) *Obstet. Gynecol.* **50**, 401–406
(5) Horwitz, C. A., Garmezy, L., Lyon, F., Hensel, M., & Burke, M. D. (1972) *Am. J. Clin. Pathol.* **58**, 305–313
(6) Chattoraj, S. (1976) in *Fundamentals of Clinical Chemistry*, N. W. Tietz (Ed.), W. B. Saunders Co., Philadelphia, PA, pp. 699–823
(7) Wide, L. (1962) *Acta Endocrinol.* **41**, *Suppl. 70*, 1–111
(8) Kerber, I. J., Inclan, A. P., Fowler, E. A., Davis, K., & Fish, S. A. (1970) *Obstet. Gynecol.* **36**, 37–43
(9) Horwitz, C. A., Maslansky, R., Waldinger, R., Cabrera, H., & Ward, P. C. J. (1973) *J. Reprod. Fertil.* **33**, 489–493

COSMETICS

Gas Chromatographic Determination of Synthetic Musk (7-Acetyl-6-Ethyl-1,1,4,4-Tetramethyltetralin) in Fragrances

HARRIS H. WISNESKI, RONALD L. YATES, and HENRY M. DAVIS
Food and Drug Administration, Division of Cosmetics Technology, Washington, DC 20204

A gas chromatographic method has been developed for the determination of the synthetic fragrance musk, 7-acetyl-6-ethyl-1,1,4,4-tetramethyltetralin (AETT). The fragrance is first added to water and extracted with ether to separate the fragrance oil from the water-alcohol mixture. The AETT, present in the ether-soluble fraction, is purified further by preparative high pressure liquid chromatography (HPLC). The AETT in the collected HPLC fraction is then determined by gas chromatography using external standardization. Recoveries from samples spiked with AETT at levels ranging from 100 to 1000 μg/mL varied from 80 to 96% with an average of 90%. AETT was verified in each sample by gas chromatography/mass spectrometry.

7-Acetyl-6-ethyl -1,1,4,4- tetramethyltetralin (AETT) is a synthetic fragrance material which was widely used until recently as a fixative in fragrances found in cosmetics, toiletries, and perfumes. In 1977 it was reported that a metabolite of AETT has a potential for producing neurotoxic effects and/or generalized tissue discoloration in several species of laboratory animals (1-3).

A survey of the literature indicated that the identification and determination of AETT in finished cosmetic products have not been investigated. Initial investigations that were conducted by our laboratory and were directed toward the detection and determination of AETT used either gas chromatography (GC) or high pressure liquid chromatography (HPLC). We found that most commercial fragrance compositions were so complex that neither method was able to resolve AETT from various interferences. After AETT along with the other carbonyl compounds was isolated from the fragrances by Girard T reagent, these mixtures were still too complex for direct determination by GC or HPLC.

Received September 22, 1981. Accepted November 16, 1981.
This paper was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

The proposed method uses preparative HPLC to fractionate the fragrance oil before determination by GC. The fragrance oil is first extracted from the cosmetic product with ether, and then the AETT contained in an aliquot of the extract is separated by preparative HPLC. The AETT fraction is collected from the HPLC column, concentrated, and diluted to a known volume. An aliquot of this solution is analyzed by GC and the amount of AETT is determined from a standard calibration curve. The method was verified by conducting recovery studies on a number of commercial fragrance compositions. A gas chromatographic/mass spectrometric (GC/MS) system for identification of AETT was also developed. AETT identity was verified by GC/MS in each of the samples used in the recovery studies.

Experimental

Apparatus

(a) *High pressure liquid chromatograph.*—Equipped with UV detector (254 nm) and gradient elution capability. Waters Model 244 with two Model 6000 A solvent pumps, Model U6K universal detector, Model 440 UV detector with 254 nm filter and Model 660 solvent programmer (Waters Associates, Inc., Milford, MA 01757), or equivalent. Operating conditions: solvent program, linear, 30 min, 20 to 80% B; solvent flow rate, 4 mL/min.

(b) *HPLC column.*—Partisil-M-9 10/50 PAC, 500 × 9.4 mm id, Cat. No. 4230-226 (Whatman, Inc., Clifton, NJ 07014), or equivalent.

(c) *HPLC guard column.*—75 × 4.6 mm id with in-line filter (10 μm porosity), packed with Corasil Type II, 37-50 μm (Waters Associates, Inc.), or equivalent.

(d) *Gas chromatograph.*—Model 3920 B equipped with flame ionization detector (Perkin-Elmer Corp., Norwalk, CT 06856), or equivalent. Column: 6 ft × 1/4 in. od, glass, packed with 80-100 mesh Gas Chrom R coated with 3% polyethylene glycol (PEG) 20M, 0.2% Alkaterge C, and 0.2% Span 80. Operating conditions:

0004-5756/82/6503-0598-04$01.00

carrier gas (helium) flow rate, 80 mL/min; column temperature, 170°C; injector and interface temperature, 220°C.

(e) *Recorder (HPLC).*—10 in. strip chart with 10 mV input.

(f) *Recorder (GC).*—10 in. strip chart with 1 mV input.

(g) *Gas chromatograph/mass spectrometer (GC/MS) system.*—Hewlett-Packard Model 5992 A (Hewlett-Packard Co., Palo Alto, CA 94304). GC column: 180 cm × 2 mm id, glass, packed with 3% SP-2100 on 100–120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA 16823). GC operating conditions: column temperature, 180–210°C at 10°/min; injection temperature, 250°C; flow rate (helium), 30 mL/min. MS operating conditions: MS peak detection threshold, 5 linear counts; sample/0.1 amu, 4; scan speed, 380 amu/s; electron multiplier, 2600 V; total abundance, from 45 to 350 amu.

(h) *Volumetric flask, micro.*—2 mL. (No. K-297000, Kontes Co., Vineland, NJ 08360, or equivalent.)

Reagents

(a) *HPLC solvents.*—Isooctane, methylene chloride, and acetonitrile. Spectrophotometric or HPLC grade. (Distilled in glass, Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442, or equivalent.) (*1*) *Solvent A.*—Isooctane; degas and filter. (*2*) *Solvent B.*—Isooctane–methylene chloride–acetonitrile (350 + 150 + 3). Degas and filter. All other solvents and reagents were ACS grade or equivalent.

(b) *7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin (AETT).*—Versalide extra. Givaudan Corp., Clifton, NJ 07014.

(c) *Standard solutions.*—(*1*) *Stock solution.*—0.5 mg/mL. Accurately weigh ca 50 mg AETT in 10 mL beaker. Dissolve in isooctane and transfer quantitatively to 100 mL volumetric flask. Dilute to volume with isooctane, and mix. (*2*) *Working solution A.*—0.05 mg/mL. Pipet 10 mL stock solution into 100 mL volumetric flask, dilute to volume with isooctane, and mix. (*3*) *Working solution B.*—0.025 mg/mL. Pipet 5 mL working solution A into 10 mL volumetric flask, dilute to volume with isooctane, and mix. (*4*) *Working solution C.*—0.005 mg/mL. Pipet 1.0 mL working solution A into 10 mL volumetric flask, dilute to volume with isooctane, and mix.

Preparation of Sample

Pipet 5.0 mL fragrance into 100 mL separatory funnel containing 15 mL water. Extract with two 15 mL portions of ethyl ether. Combine ether extracts and wash with two 20 mL portions of water. Dry ether extract over anhydrous Na_2SO_4 for 2 h. Transfer dried ether extract to a 50 mL tapered-tip centrifuge tube. Add a boiling chip and evaporate carefully on steam bath to ca 2 mL. Add 1–2 mL isooctane and concentrate under gentle air jet to 1–1.5 mL. Using a syringe, transfer to a 2.0 mL volumetric flask. Rinse centrifuge tube with several small portions of isooctane and add to volumetric flask. Dilute to volume with isooctane and mix. If solution appears cloudy, add small amount of anhydrous Na_2SO_4. Retain solution for preparative HPLC.

Using conditions outlined under (a) *High pressure liquid chromatograph*, obtain analytical chromatogram of AETT by injecting 10 μL of stock solution at 0.2–0.5 AUFS. Repeat until repetitive retention times are obtained (±45 s). Obtain an analytical chromatogram of the sample by injecting 10 μL at 0.1–0.2 AUFS. Compare chromatogram with that of the AETT standard to determine which peak corresponds to AETT. If there is any difficulty determining which peak corresponds to AETT, spike 10 μL of sample with 5–10 μL of AETT stock solution and obtain an additional analytical chromatogram of the sample. After locating AETT peak, change absorbance setting appropriate to a larger sample and inject an accurately known volume of ca 100 μL of sample solution. Collect eluate over the predetermined retention volume range of AETT. Concentrate eluate on steam bath under a gentle air jet to ca 2 mL and quantitatively transfer to a 5.0 mL volumetric flask. Dilute to volume with isooctane, and mix.

Determination

Using conditions outlined under (d) *Gas chromatograph*, obtain gas chromatogram of AETT by injecting several microliters of stock solution. Obtain analytical chromatogram of sample to identify peak corresponding to AETT. Inject an accurately known volume of 6–8 μL of working solution A into gas chromatograph, using appropriate attenuation to keep peak 50–90% full scale. Similarly, obtain GC data for working solutions B and C. Inject an accurately known volume of 6–8 μL of sample, adjusting attenuation to keep AETT peak at 50–90% full scale. Obtain 3 sets of data for each working standard and sample.

Calculation

For each working standard, measure peak heights and calculate average. Correct averaged

peak heights to a common attenuation and plot μg AETT vs peak heights. Obtain average peak height of sample, correct to attenuation of standards, and obtain μg AETT from calibration curve. Calculate C, concentration of AETT in sample (μg/mL):

$$C = V_E \times V_G \times M_X/(V_H \times V_X \times V_S)$$

where V_E = mL sample extract (2.0 mL); V_G = mL HPLC eluate (5.0 mL); M_X = μg AETT from calibration curve; V_H = mL sample extract injected into high pressure liquid chromatograph; V_X = mL HPLC eluate injected into gas chromatograph; V_S = mL sample.

If concentration of AETT is to be determined in μg/g, weigh sample before extraction and substitute M_s (g) for V_s (mL) in above equation.

Confirmation

Using conditions given under (g) *GC/MS system*, inject a volume of the final sample solution that contains 100–200 ng AETT. Confirm identity of AETT by comparing mass spectrum of sample with that of a standard.

Results and Discussion

Fragrances are complex mixtures of natural and synthetic ingredients, typically containing over 100 compounds. Initial investigations employing HPLC and GC (packed and wall-coated open tubular columns) demonstrated that neither technique alone was able to resolve AETT from interferences. To reduce the complexity of the mixture to be analyzed, AETT and other carbonyl compounds were separated as a group from the other fragrance compounds with Girard T reagent. After regeneration, the carbonyl fraction was analyzed by GC and by HPLC, but it was still too complex for the separation of AETT from other interfering ingredients. From these preliminary investigations, it was apparent that the components in the AETT fraction would have to be separated further before chromatographic analysis to remove interfering substances. Preliminary separation experiments with the isolated fragrance oil, using preparative HPLC followed by GC analysis, indicated that adequate separation of interferences could be obtained.

When preparative HPLC is used as a sample cleanup procedure with relatively nonpolar HPLC solvents, it is important that the sample contain no polar solvents or diluents. Fragrance compositions, however, contain large amounts of alcohol with smaller amounts of water. If injected into the HPLC chromatograph, alcohol and water cause changes in retention volume and loss of resolution, which in turn require the frequent use of column reactivation procedures. To minimize these adverse effects, most of the alcohol and water are removed from the fragrance by extraction with ether followed by washing the extract with water. Although most polar, water-soluble compounds are extracted from the fragrance oil, slight variations can be expected in the AETT retention volumes of the standard and the sample. These variations are more pronounced when preparative runs are made, especially when gradient elution is used. In complex samples having a low AETT level, it may be difficult to establish the definite location of the AETT peak by retention data alone. For this reason, it is recommended that analytical chromatograms of the unspiked sample and the sample spiked with AETT be obtained to resolve any ambiguities. Dead volume between detector and collection port should be taken into consideration in determining when to start and stop eluate collection.

After several preparative runs, the HPLC column may exhibit decreased retention volumes, an unstable baseline, and some loss of resolution. At this point the column should be washed with polar solvents and reactivated by reconditioning with dry isooctane. The column manufacturer usually supplies specific directions for accomplishing this. We have found that complete reconditioning is needed only after long use. However, because of the relatively large quantity of sample analyzed, it is good practice to wash the column with a more polar solvent after the analysis is completed to elute remaining sample components. The wash solvent should be able to accomplish this without significantly affecting column activity. Initial efforts using wash solvents containing moderate and low levels of methyl or isopropyl alcohol adversely affected column activity and retention volume reproducibility, even after prolonged equilibration at initial conditions. We found that the column can be restored to good working conditions by washing with 250–300 mL of methylene chloride–acetonitrile (25 + 1) followed by 250 mL of initial HPLC solvent (80% A + 20% B). Flow rate can be increased to 6–8 mL/min to reduce the time required.

It is important to reduce the volume of the collected HPLC eluate to approximately 2 mL before dilution to volume to ensure that nearly all of the methylene chloride has been removed. Methylene chloride tends to produce a long,

Table 1. Recoveries of AETT from spiked commercial fragrances

Sample	AETT added, μg/mL	AETT recovered μg/mL	AETT recovered %
1	1000	910	91
		900	90
		900	90
2	1000	910	91
		920	92
		920	92
3	1000	910	91
		920	92
		920	92
4	500	460	92
		470	94
		460	92
5	500	460	92
		460	92
		470	94
6	500	480	96
		470	94
		480	96
7	100	87	87
		87	87
		85	85
8	100	94	94
		93	93
		88	88
9	100	80	80
		8[illegible]	81
		8[illegible]	81
Av.			90

tailing, solvent peak on the gas chromatogram, which may prevent reduction of attenuation to the proper range.

Ten commercial fragrance oils were selected for recovery studies. One of the 10 was rejected because it contained AETT. The fragrance compositions were prepared by adding known amounts of AETT to 10 mL of fragrance oil and diluting to 100.0 mL with ethanol. The recovery data are shown in Table 1. All samples were analyzed by GC/MS, and in all cases the presence of AETT was confirmed.

Conclusion

A method has been developed for the GC determination and GC/MS verification of AETT in complex fragrance compositions. Preparative HPLC is used for efficient sample cleanup. The accuracy of the method was verified by recovery studies conducted with 9 commercial fragrance oils. The average of all recoveries is 90%.

Acknowledgment

The authors thank Hardy Chou, Division of Cosmetics Technology, for GC/MS verification of AETT in the samples studied.

References

(1) Spencer, P. S., Sterman, A. B., Horoupian, D., Bischoff, M., & Foster, G. (1979) *Neurotoxicology* **1**, 221

(2) Opdyke, D. L. (1979) *Food Cosmet. Toxicol.* **17**, 357–390

(3) Opdyke, D. L. (November 7, 1977) *Advisory Letter No. 16*, Research Institute for Fragrance Materials, Inc., 375 Sylvan Ave., Englewood Cliffs, NJ 07632

MICROBIOLOGICAL METHODS

Detection of Invasiveness of Mammalian Cells by *Escherichia coli*: Collaborative Study

IRA J. MEHLMAN, AIDA ROMERO, JOHN C. ATKINSON,[1] CALVIN AULISIO, and ARVEY C. SANDERS
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Collaborators: W. Campbell; J. Cholenski, J. Ferreira; E. Forney; K. O'Brian; M. Palmieri; S. Weagant

In a collaborative study of 2 standard procedures (Procedure I, presented in *J. Assoc. Off. Anal. Chem.* 60, 546-562 (1977) and Procedure II in the *Bacteriological Analytical Manual*, 1978), 7 laboratories evaluated the invasive potential of *Escherichia coli*. Monolayers of HeLa cultures were infected with *E. coli* (infectivity ratio 100 bacteria/mammalian cell) suspended in 2 menstrua: heat-inactivated fetal bovine serum (Procedure I) and 0.2% bovine albumin Fraction V dissolved in Earle's buffered salts (Procedure II). After uptake of the bacteria, intracellular growth, and differential staining, the percentages of HeLa cells containing a minimum of 5 bacteria were determined microscopically. All laboratories correctly identified invasiveness of *E. coli* by Procedure I; 5 of 6 laboratories recognized invasiveness by Procedure II. Although Procedure I was more reproducible than Procedure II, repeatability did not differ significantly. Procedure I was adopted official first action for presumptive recognition of invasiveness in *E. coli*.

Invasive *Enterobacteriaceae* invade the epithelial cells of the small and large intestines, growing intracellularly or using the cells for passage across the gastrointestinal barrier and into the blood or lymph to produce a disseminated infection (1). The classical test for invasiveness of *Escherichia coli* and *Shigella* spp. involves the production of diarrhea and/or dysentery in primates after administration in food or fluids (2–4). A more practical test is the Sereny reaction, in which keratoconjunctivitis follows topical application of a heavy bacterial suspension in the conjunctival sac of the guinea pig (5–7). This reaction gives results equivalent to those obtained in primate feeding studies for *E. coli*. Routine use of animals for estimation of pathogenicity, however, is precluded by resources, time factors, and potential danger to personnel.

In previous studies (8–14), pathogenic strains of invasive bacteria penetrated a variety of mammalian cell lines and multiplied intracellularly. Routine use of cell cultures, however, was hindered by the difficulty in distinguishing intra- from extra-cellular bacteria and the loss of the monolayer, which resulted from the microbial production of cytotoxin. To minimize these difficulties, we developed a procedure (15), known here as Procedure I, which recommended (*1*) controlled conditions for preparation of the monolayer; (2) optimal growth conditions for the bacteria; (3) control of the infection phase (i.e., infectivity ratio, length of contact, and medium); and (4) an intracellular phase. The procedure was developed for *E. coli*, *Shigella* spp., *Salmonella* spp., *Yersinia enterocolitica*, and *Y. pseudotuberculosis*. In later efforts, another procedure, known here as Procedure II, was found to be effective for *Salmonella* and *Yersinia* species (16). This study evaluated the repeatability and reproducibility of both procedures for detection of the invasiveness of mammalian cells by *E. coli*.

Escherichia coli
Detection of Invasiveness
Official First Action

46.C01 ***Principle***

Invasiveness is detected by intracellular growth on monolayer of HeLa cells on slides. To minimize extracellular bacterial multiplication, host-pathogen interaction is resolved into 2 phases, infective and intracellular, using appropriate substrates and the following protocols:

Received June 22, 1981. Accepted September 11, 1981.
This report of the Associate Referee, I. J. Mehlman, 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 F and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* 65, 000-000 (1982).

[1] Division of Mathematics.

0004-5756/82/6503-0602-06$01.00

growth of monolayer in chamber slides, using controlled inoculum and period of incubation; detn of optimal pre-infection growth conditions for pathogen; washing pathogen to remove toxic end products; infection of host cell under controlled conditions of number and multiplicity of infection, and medium and length of incubation; subsequent removal of unattached bacteria; use of post-infection medium to permit only intracellular bacterial growth for limited period.

46.C02 *Culture Media*

(a) *Minimal essential medium (MEM).*—Eagle-type with Earle's salts. Dissolve 126.4 mg L-arginine.HCl, 24 mg L-cystine, 292 mg L-glutamine, 41.9 mg L-histidine.HCl.H_2O, 52.5 mg L-isoleucine, 52.4 mg L-leucine, 73.1 mg L-lysine.HCl, 14.9 mg L-methionine, 33.0 mg L-phenylalanine, 47.6 mg L-threonine, 10.2 mg L-tryptophan, 36.2 mg L-tyrosine, 46.8 mg L-valine, 1 mg D-calcium pantothenate, 1 mg choline chloride, 1 mg folic acid, 2 mg isoinositol, 1 mg pyridoxal HCl, 1 mg nicotinamide, 0.1 mg riboflavin, 1 mg thiamine.HCl, 1 g glucose, 265 mg $CaCl_2$. $2H_2O$, 400 mg KCl, 200 mg $MgSO_4.7H_2O$, 6.8 g NaCl, 2.2 g $NaHCO_3$, 140 mg $NaH_2PO_4.H_2O$, and 10 mg phenol red in 1 L H_2O. Sterilize by filtration. Final pH should be 7.2 ± 0.2. Check sterility of all culture fluids before use. Store at 4–8°.

(b) *Fetal bovine serum (FBS).*—Sterile, virus-screened, mycoplasma-free, obtained aseptically during slaughter (Flow Laboratories, Inc., McLean, VA 22102). Store at 4–8°.

(c) *Antibiotic concentrate (AC).*—Dissolve 500 000 international units (IU) penicillin G and 500 mg streptomycin (Flow Laboratories, Inc.) in 100 mL H_2O and sterilize by filtration. Store at −10°.

(d) *MEM–FBS–AC medium.*—Routine medium for cultivation of HeLa mammalian cells. Mix 90 mL MEM (a), 10 mL FBS (b), and 1 mL AC (c). Store at 4–8°.

(e) *MEM–FBS medium.*—Medium for cultivation of HeLa cells before infection. Mix 90 mL MEM (a) and 10 mL FBS (b). Store at 4–8°.

(f) *Earle's salts.*—Prep. without phenol red as follows: Dissolve 6.8 g NaCl, 400 mg KCl, 265 mg $CaCl_2$, 200 mg $MgSO_4.7H_2O$, 140 mg $NaH_2PO_4.H_2O$, 1.0 g glucose, and 2.2 g $NaHCO_3$ in 1 L H_2O. Sterilize by filtration. Final pH should be 7.2 ± 0.2.

(g) *Veal infusion broth.*—Dissolve 500 g veal (infusion) and 10 g proteose peptone in 1 L H_2O with gentle heating. Dispense 5 mL portions into 13 × 100 mm screw-cap tubes. Autoclave 15 min at 121°. Final pH should be 7.3 ± 0.2.

(h) *Brain–heart infusion (BHI).*—Dissolve 12.5 g BHI (powder) in 1 L Earle's salts (f). Sterilize by filtration. Final pH should be 7.2 ± 0.2.

(i) *Bile salts No. 3.*—Dissolve 5 g bile salts No. 3 formulation in 1 L Earle's salts (f). Sterilize by filtration.

(j) *Heat-inactivated HFBS.*—Heat FBS (b) 2 h at 55 ± 1°. Store at 4–8°.

(k) *HFBS–BHI–BS medium.*—Mix 20 mL heat-inactivated FBS (j), 10 mL BHI (h), 10 mL bile salts No. 3 (i), and 60 mL Earle's salts (f). Store at 4–8°.

(l) *Veal infusion agar slant.*—For maintenance of cultures. Dissolve 500 g veal (infusion), 10 g proteose peptone No. 3, 5 g NaCl, and 15 g agar in 1 L H_2O with gentle heating. Dispense 7 mL aliquots to 16 × 150 mm screw-cap tubes. Autoclave 15 min at 121°. Final pH should be 7.3 ± 0.2.

(m) *Dulbecco's phosphate-buffered saline (PBS).*—Dissolve 8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4, 100 mg $CaCl_2$, and 100 mg $MgCl_2.6H_2O$ in 1 L H_2O. Sterilize by filtration. Final pH 7.2 ± 0.2.

(n) *Calcium- and magnesium-free Dulbecco's PBS.*—Dissolve 8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, and 200 mg KH_2PO_4 in 1 L H_2O. Sterilize by filtration. Final pH 7.2 ± 0.2.

(o) *Calcium, magnesium, phenol red-free Hank's PBS.*—Dissolve 8.0 g NaCl, 400 mg KCl, 90 mg $Na_2HPO_4.7H_2O$, 60 mg KH_2PO_4, 1.0 g glucose, and 350 mg $NaHCO_3$ in 1 L H_2O. Sterilize by filtration. Final pH 7.2 ± 0.2.

(p) *Trypsin stock soln.*—2.5%. Suspend 2.5 g 1:250 trypsin (Difco Laboratories) in 100 mL Ca- and Mg-free Hanks' PBS (o) and let particles settle. Sterilize by filtration. Dil. 10 mL stock soln with 90 mL sterile Ca- and Mg-free Dulbecco's PBS (n) to prep. 0.25% trypsin. Store at −10°.

(q) *Gentamicin stock soln.*—Dissolve 50 mg gentamicin (Schering Corp., Kenilworth, NJ 07033) in 100 mL Dulbecco's PBS (m) to give soln contg 500 μg/mL. Dil. 1 + 9 with Dulbecco's PBS to soln contg 50 μg/mL. Store at 4–8°

(r) *Lysozyme soln.*—Weigh 0.3 g lysozyme, 3× crystalline, salt-free, ca 12 000 Shugar units/mg (Calbiochem-Behring, San Diego, CA 92112), into 100 mL Dulbecco's PBS and stir to dissolve. Store at 4–8° not >2 weeks.

(s) *Intracellular growth phase medium.*—Mix 80 mL MEM–FBS medium (e), 10 mL gentamicin soln (50 μg/mL) (q), and 10 mL lysozyme soln (r). Prep. immediately before use.

46.C03 *Diagnostic Reagents*

(a) *May-Grunwald stain.*—Weigh 2.5 g stain (Matheson, Coleman & Bell) into 50 mL absolute MeOH, dissolve by grinding, and dil. to 1 L with MeOH. Stir 16 h at 37°. Hold stain 1 month at 22° (room temp.). Filter for use.

(b) *Giemsa stain.*—Dissolve 1 g stain (Matheson, Coleman & Bell) in 66 mL glycerol by heating 1.5–2.0 h at 55–60°. Add 66 mL absolute MeOH. Store stain 2 weeks in tightly stoppered bottle at 22°. Dil. stock soln (1 + 9) before use.

(c) *Decolorizing and dehydrating reagents.*—Acetone; acetone-xylene (50 + 50) and (33 + 67); xylene.

(d) *Mounting medium.*—Dil. mounting medium with xylene to give easily dispensed colloidal suspension; 20 mL Permount™ (Fisher Scientific Co.) dild with 5 mL xylene is satisfactory.

(e) *Human cervical epithelial cell culture.*—ATCC HeLa culture. Other cultures, including Henle 407 human intestine and human laryngeal carcinoma gave comparable data; however, HeLa cell culture was more suitable with regard to culture characteristics.

46.C04 *Apparatus*

(a) *Water baths.*—Maintained at 35 ± 1° and 55 ± 1°.

(b) *Microscopes.*—Standard 900X magnification; inverted stage, 100X magnification (Preiser Scientific, Charleston, WV 25322), or equiv.; microscope illuminator.

(c) *Carbon dioxide incubator.*—95% air–5% CO_2-moisture-satd atmosphere, maintained at 36 ± 1° (Lab-Line Instruments, Inc., Melrose Park, IL 60160, or equiv.).

(d) *Tissue culture chamber slides.*—Clean microscope slides mounted with partitions on plastic gasket to facilitate multiple testing. Lab-Tek units contg 4 chambers are satisfactory (Miles Laboratories, Inc., Naperville, IL 60540), or equiv.

(e) *Culture containers.*—Sterile 3 fluid oz (85 mL) glass prescription bottles or plastic tissue culture flasks (Costar, Cambridge, MA 02139, or equiv.).

(f) *Glass cover slips.*—1 × 2 in. (2.5 × 5.1 cm).

(g) *Cell-counting chamber.*—Spencer Bright Line, Fuchs-Rosenthal (Preiser Scientific), or equiv.

(h) *Refrigerated centrifuge with adapter.*—To accommodate 13 × 100 mm tubes and covered centrf. cups to prevent aerosolization of pathogens.

(i) *Membrane filters.*—0.45 μm pore diam. (Millipore Corp., Bedford, MA 01230, or equiv.).

46.C05 *Preparation of HeLa Cell Culture*

Using std cell culture technics, grow HeLa strain on inner surface of 3 oz glass or plastic container, using 5 mL MEM-FBS-AC medium, (d), for 7 days at 36° in CO_2 incubator. Replace with fresh culture medium on fourth day to prevent accumulation of toxic metabolites. In prepg cells in monolayer for transfer to chamber slides, wash once with 5 mL Dulbecco's PBS (m) prewarmed at 36°. Add 5 mL prewarmed (36°) 0.25% trypsin and hold at room temp. 2 min. Aseptically remove ca 4.5 mL trypsin. Incubate flask at 36° with occasional agitation. After monolayer has detached and cells are fairly uniformly distributed in residual trypsin, add 25 mL prewarmed (36°) MEM-FBS medium, (e). Est. cell density, using counting chamber. Add MEM-FBS medium, if necessary, to dil. suspension to density of 1×10^5 cells/mL. With occasional agitation, rapidly transfer 1 mL aliquots to chambers of slide. Incubate 20–24 h at 36° in CO_2 incubator. Aseptically remove spent medium before infection. Wash each monolayer once with 1 mL prewarmed (36°) Earle's salts, (f), and 1 mL prewarmed (36°) uninoculated infection medium, (k) (see below).

46.C06 *Preparation of Bacteria*

Inoculate, with needle, 5 mL veal infusion broth, (g), using growth from veal infusion agar slant (l) incubated at 22°. Incubate presumptive *E. coli* broth cultures 18–24 h at 36°. Centrf. suspension 20 min at 1200X g at 18°. Resuspend cells in equal vol. of Earle's salts, (f). Recentrifuge 20 min at 1200X g. Resuspend cells in 5 mL Earle's salts. Dil. latter suspension with prewarmed (36°) HFBS-BHI-BS medium, (k), to final density of 5×10^7 cells/mL. Add 0.2 mL of each suspension to prepd chamber (above). Use 0.2 mL HFBS-BHI-BS for uninoculated neg. control.

46.C07 *Infection Stage*

Incubate chambers 2.5 h at 36° in CO_2 incubator. Time factor is critical; shorter period results in min. number of infected host cells and longer period may result in cytotoxic effect arising from medium and possibly bacterial metabolites.

46.C08 *Intracellular Growth Stage*

Remove infection medium from chamber with Pasteur pipet. To prevent contamination, use

sep. pipet for each chamber. Wash each chamber twice with 1 mL aliquots of prewarmed (36°) Earle's salts. Subsequently wash with 1 mL aliquot of prewarmed intracellular growth phase medium (s) prepd immediately before use. Add 0.8 mL prewarmed intracellular growth phase medium to each chamber. Incubate 5 h at 36° in CO_2 incubator. Control of extracellular growth is critical at this stage; sensitivity of culture to gentamicin and other antibiotics should be examined by std procedures before pathogenicity testing. Problem is critical in meats and dairy products where antibiotics may have been used in therapy or in feeds.

46.C09 ***Staining***

Remove fluid contents of chambers. Wash monolayer 3 times with 1 mL Dulbecco's PBS (**n**). Add 1 mL absolute MeOH fixative per chamber. Hold at room temp. 5 min. Remove MeOH and side walls of chamber slide. Insert single-edge razor blade between gasket and slide, and gently pry gasket from slide. If necessary, cautiously remove remnants of gasket from slide with razor blade. Do *not* let specimen dry while slide is prepd for staining. Immerse slides in May-Grunwald stain (**a**) 10 min. Withdraw slides, remove excess stain, and immerse in Giemsa stain (**b**) 20 min. Withdraw slides, remove excess stain, and immerse in H_2O 10–20 s. Briefly rinse twice in acetone. Briefly immerse slides in following sequence of solvs: acetone–xylene (50 + 50), acetone–xylene (33 + 67), and xylene. Evenly distribute 4 drops of mounting medium, (d) to slide. Place large cover slip on prepn. Remove excess mounting medium and xylene by gently blotting. Gently apply pressure to remove air bubbles from prepn.

46.C10 ***Detection and Criteria of Invasiveness***

Examine specimens with 900× magnification. Criterion for intracellular location of bacteria is parfocality of cytoplasmic ground substance and bacteria. If invasive, *E. coli.* occur within cytoplasm. Frequently, they may be located along nuclear membrane. In addition, they may be elongated. Finally, bacteria may occur within a membrane (phagolysosome) individually or in groups, indicative of intracellular growth. Examine, at random, 10 fields contg 15–25 HeLa cells. Count bacteria in each cell. Criterion for infection is ≥5 bacteria per cell. Criterion for invasiveness of bacterial culture is ≥1.0% infected HeLa cells.

HeLa cells results with *E. coli* strains must be confirmed by Sereny keratoconjunctivitis test (1–3).

SELECTED REFERENCES

(1) *Acta Microbiol. Acad. Sci. Hung.* **2,** 292 (1955).
(2) *Acta Microbiol. Acad. Sci. Hung.* **4,** 367 (1957).
(3) *J. Hyg. Epidemiol. Microbiol. Immunol.* **3,** 292 (1959).

Results and Discussion

The collaborative study originally included 7 participating laboratories. However, because one laboratory reported only qualitative data, this paper includes only data from the other 6 laboratories (Table 1). All laboratories identified the invasiveness of *E. coli* by Procedure I. Laboratory D was unable to recognize invasive potential by Procedure II in the 5 strains provided. Noninvasiveness was correctly identified in all strains by all laboratories using both procedures; however, laboratory data varied considerably, with some participants tending to obtain consistently high or low values. Using the null difference hypothesis that the laboratory is not a variable, the F values (ratio = laboratory mean square/error mean square) for Procedures I and II were 31.6 and 71.6, respectively (17). The probability of this occurrence by chance is <1/10 000. No difference was noted between the procedures for repeatability within a laboratory, based on the calculation of error mean squares with 54 degrees of freedom for all variables. The values were 0.00227 and 0.00329 with an 8.8% probability of this difference to occur. With respect to reproducibility, the statistical parameter was the variance component among laboratories plus the repeatability value. The values for Procedures I and II were 0.0092 and 0.0264, respectively. Thus, Procedure I is more reproducible than Procedure II.

Finally, the Duncan analysis (18) confirmed the earlier observation of consistent trends among the laboratories to report high or low values (Table 2). Using Procedure I, the 6 laboratories could be ranked in 5 groups, each reporting values significantly different from the other laboratories. Using Procedure II, the laboratories could be ranked in 4 groups. Laboratory C tended to report high values using both techniques, whereas laboratory A tended to report low values. These data suggest the existence of controlled and uncontrolled variables. The former may include interpretation of microscopic data; the latter may include variability of the media used for growing the bacteria and mammalian cells and for infection. These factors should be emphasized in training sessions

Table 1. Percentage of HeLa cells infected by *Escherichia coli*

	Laboratory											
	A		B		C		D		E		F	
Culture	I[a]	II[b]	I	II	I	II	I	II	I	II	I	II
1(−)[c]	0,0[d]	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
2(−)	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
3(+)[e]	33,33	14,18	3,6	51,57	94,98	94,98	51,90	0,2	22,28	77,78	52,58	58,63
4(−)	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
5(+)	5,12	32,35	1,3,3,4	39,47	99,100	99,100	53,70	0,0	40,40	76,76	28,51	43,57
6(+)	23,24	19,20	4,5,6,1	26,49	98,100	98,100	70,70	0,1	21,26	75,76	39,62	71,79
7(−)	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
8(+)	15,21	11,19	2,3,3,2	47,60	100,100	100,100	52,65	1,2	31,32	84,93	15,32	35,56
9(−)	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
10(+)	10,10	21,24	10,13	43,57	95,100	95,100	40,76	0,1	28,29	86,95	18,21	38,50

[a] I = AOAC procedure.
[b] II = BAM procedure.
[c] (−) = Noninvasive.
[d] Duplicate determinations.
[e] (+) = Invasive.

and in the development of other tests for pathogenicity.

Conclusion

This study represents the second attempt of the Food and Drug Administration to conduct a collaborative study for standardization of pathogenicity tests. The first sought confirmation of the reliability and validity of the methodology in which *Clostridium botulinum* toxins in food (19, 20) were detected through an interaction between the toxins and a living animal system, the mouse. The test used in the present study involves an interaction of viable bacterial cells and mammalian cell cultures. It is likely that within the near future, other complex tests for attributes of pathogenicity will be developed and recommended for adoption, with statistical analysis needed to evaluate their repeatability and reproducibility. Variability will probably be encountered more often in pathogenicity testing than in other microbiological assays because pathogenicity testing involves 3 systems: the pathogen, the uninfected host, and the infected host.

Based on the results of this collaborative study, we recommend that Procedure I be adopted official first action for presumptive recognition of invasiveness of *E. coli*. HeLa cell data, however, must be confirmed by the Sereny reaction.

Acknowledgment

The authors thank the following participants, all of FDA: Warren Campbell, Los Angeles, CA (present address, National Center for Toxicological Research, Jefferson, AR); James Cholenski, Minneapolis Center for Microbiological Investigations, Minneapolis, MN; Joseph Ferreira,

Table 2. Logarithm percent positive as function of invasive *Escherichia coli*

Method I			Method II		
Group[a]	Laboratory	Log mean %[b]	Group	Laboratory	Log mean %
I	C	1.992894	I	C	1.992894
				E	1.909314
II	D	1.793317	II	F	1.727201
				B	1.667137
III	F	1.544549	III	A	1.305298
	E	1.463696			
IV	A	1.205543	IV	D	0 120412
V	B	0.832006			

[a] Groups based on significantly different logarithm average percent infected HeLa cells.
[b] Logarithm base of 10 of average percent infected HeLa cells obtained with 5 invasive strains.

Atlanta, GA; Evelyn Forney, Cincinnati, OH; Kevin O'Brian, Baltimore, MD; Michael Palmieri, New York, NY; Steven Weagant, Seattle, WA.

Thanks are also extended to the following individuals, who contributed cultures used in this study: S. B. Formal, Walter Reed Army Institute of Research, Washington, DC; G. Controni, Children's Hospital, Washington, DC; H. L. DuPont, University of Texas Medical School, Houston, TX; S. L. Gorbach, Tufts-New England Medical Center, Boston, MA; D. J. Brenner, Center for Disease Control, Atlanta, GA; and S. Winblad, University of Lund, Allmanna Sjukhuset, Malmo, Sweden.

REFERENCES

(1) Takeuchi, A., Formal, S. B., & Sprinz, E. (1968) *Am. J. Pathol.* **52,** 503–513
(2) Rout, W. R., Formal, S. B., Giannella, R. A., & Dammin, G. J. (1975) *Gastroenterology* **68,** 270–278
(3) DuPont, H. L., et al. (1971) *N. Engl. J. Med.* **285,** 1–9
(4) Levine, M. M., et al. (1973) *J. Infect. Dis.* **127,** 261–270
(5) Sereny, B. (1955) *Acta Microbiol. Acad. Sci. Hung.* **2,** 292–296
(6) Sereny, B. (1957) *Acta Microbiol. Acad. Sci. Hung.* **4,** 367–376
(7) Sereny, B. (1959) *J. Hyg. Epidemiol. Microbiol. Immunol.* **3,** 292–305
(8) Shepard, C. C. (1955) *Proc. Soc. Exp. Biol. Med.* **90,** 392–396
(9) Shepard, C. C. (1957) *J. Exp. Med.* **105,** 39–57
(10) Shepard, C. C. (1958) *J. Exp. Med.* **107,** 237–251
(11) Gerber, D. F., & Watkins, H. M. S. (1961) *J. Bacteriol.* **82,** 815–822
(12) Ogawa, H., Yoshikura, H., Nakamura, A., & Nakaya, R. (1967) *Jpn. J. Med. Sci. Biol.* **20,** 329–337
(13) Giannella, R. A., Washington, O., Gemski, P., & Formal, S. B. (1973) *J. Infect. Dis.* **128,** 69–75
(14) Tenner, K., Kaiserling, E., Lennert, K., Racz, P., & Wuthe, H. H. (1973) *Virchows Arch. B.* **14,** 35–46
(15) Mehlman, I. J., Eide, E. L., Sanders, A. C., Fishbein, M., & Aulisio, C. C. G. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 546–562
(16) Mehlman, I. J., & Aulisio, C. C. G. (1978) in *Bacteriological Analytical Manual*, 5th Ed., AOAC, Arlington, VA, Chapter XVIII
(17) Youden, W. J. (1969) *Statistical Techniques for Collaborative Tests*, AOAC, Arlington, VA
(18) Duncan, D. B. (1965) *Technometrics* **7,** 171
(19) Kautter, D. A., & Solomon, H. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 541–545
(20) Schantz, E. J., & Kautter, D. A. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 96–99

SUGARS AND SUGAR PRODUCTS

Mass Spectrometric $^{13}C/^{12}C$ Determinations to Detect High Fructose Corn Syrup in Orange Juice: Collaborative Study

LANDIS W. DONER and DONALD D. BILLS
U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118

Collaborators: O. Carro; R. Drimmie; P. Fritz; J. N. Gearing; C. Hillaire-Marcel; P. L. Parker; R. M. Reeseman; B. N. Smith; H. Ziegler

The $^{13}C/^{12}C$ ratios in orange juice are sufficiently uniform and different from those in high fructose corn syrup (HFCS) so that the addition of HFCS to orange juice can be detected. HFCS averages −9.7‰ (parts per thousand) $\delta^{13}C$, orange juice averages −24.5‰, and mixtures of HFCS and orange juice possess intermediate values. One pure orange juice and 4 orange juice-HFCS mixtures containing from 25 to 70% orange juice were properly classified by 7 collaborators. Samples with $\delta^{13}C$ values less negative than −22.1‰, 4 standard deviations from the mean of pure juices, can, with a high degree of confidence, be classified as adulterated. Samples with values more negative than −22.1‰ must be considered unadulterated with HFCS, because pure orange juices possess a range of $\delta^{13}C$ values. The $^{13}C/^{12}C$ mass spectrometric method was adopted official first action for detecting HFCS in orange juice.

The economic incentives to adulterate orange juice with less expensive solutions of sugars are significant, and previous methods developed to indicate such adulteration have been reviewed (1–3). Orange juice is also adulterated by addition of orange pulpwash (water-extracted soluble orange solids from orange pulp), and recently an effective spectrophotometric method was developed to detect such adulteration (4). A microbiological assay procedure has been developed (5) which indicates the content of orange juice in a product; the versatility of this latter method has been demonstrated by extension to other food adulteration problems, such as detecting grape juice added to apple juice (6).

The present report demonstrates the application of stable carbon isotope ratio analysis (SCIRA) for detecting illegal addition of high fructose corn syrup (HFCS) to orange juice. Sugar analysis is not suitable for this purpose, because HFCSs are available which, when added to orange juice, will result in glucose and fructose levels within the range found for authentic juices. We previously applied SCIRA to the detection of HFCS in honey (7) and apple juice (8), and the AOAC official first action methods are widely used. The SCIRA approach takes advantage of the fact that organic materials derived from C_4 plants such as corn have elevated $^{13}C/^{12}C$ ratios compared with those derived from C_3 plants such as apple trees and all honey floral sources. We recently reported (9) that pure orange juices (42 samples) from several locations are extremely uniform in $\delta^{13}C$, averaging −24.5‰ with a coefficient of variation of 2.41%. The orange juice samples included domestic blends, samples of varietal purity, and foreign samples. The results agreed with earlier analyses by Nissenbaum et al. of Israeli and French juices (10). Being derived from a C_4 plant, HFCS is richer in ^{13}C, with $\delta^{13}C$ values averaging −9.7 (7).

Experimental

Materials

Pure orange juice was squeezed from oranges of the Navel variety and determined to contain 11.60% solids. Navel orange juice was chosen because its $\delta^{13}C$ values have been shown (9) to fall in the range of values for pure juices. HFCS was obtained from Corn Sweeteners Co. (Cedar Rapids, IA), and diluted to 11.60% solids with water. Orange juice and HFCS were then combined to give adulterated mixtures containing the following proportions of orange juice: A, 70.0%; B, 25.0%; C, 100.0%; D, 40.0%; and E, 55.0%. These 5 samples were freeze-dried, and

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 August 28, 1981. Accepted September 9, 1981.
This report of the Associate Referee, L. W. Doner, 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 D and was adopted by the Association. See the report of the committee, March 1982 issue, for detailed recommendations.

0004-5756/82/6503-0608-03$01.00

Table 1. Instruments and conditions used in collaborative study

Coll.	Mass spectrometer	Combustion[a] Temp., °C	Combustion[a] Recirc. time, min	Coll. study working std
1	Micromass 602 D	600	sealed tube with CuO	AER vacuum pump oil
2	Micromass 602 D	850	10	charcoal ($\delta^{13}C = -23.3‰$ vs PDB) and marble ($\delta^{13}C = 0.0‰$ vs PDB) calibrated against NBS-20 solenhofen limestone ($\delta^{13}C = -29.5‰$ vs PDB)
3	Micromass 602 C	1200	—	UQ2 marble vs NBS-22 and PDB
4	Nier type, 6 in. 60° sector, dual collecting	810	20	BYU carbonate calibrated against NBS-22, NBS-oxalate, and NBS-21
5	Micromass 602	>1500	—	bender limestone
6	Mat 250, Varian	750	—	flask CO_2 vs PDB
7a	Micromass 903	—	—	tank CO_2

[a] Sample combustion in Parr bomb, electrically ignited after charging with 12 atmospheres O_2.

portions were added to screw-top vials labeled A through E and sent to collaborators.

Sample Combustion and Determination of $\delta^{13}C$

Various isotope ratio mass spectrometers are used in laboratories which determine $^{13}C/^{12}C$ ratios. Also, different procedures are used to quantitatively burn the sample to carbon dioxide and water. Overall accuracy, including combustion and $^{13}C/^{12}C$ determination, is 0.3‰ or better. The instruments, combustion conditions, and standards used by the collaborators are given in Table 1. $\delta^{13}C$ (‰) values are reported from comparisons with carbon dioxide generated from the reference standard PeeDee belemnite (PDB) and calculated from the following formula after comparison of the $^{13}C^{16}O_2$ and $^{12}C^{16}O_2$ ion beams:

$$\delta^{13}C\ (‰) = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{PDB}}\right] - 1 \times 10^3$$

A $\delta^{13}C$ value of −25‰ means that the $^{13}C/^{12}C$ ratio of the sample is 2.5% less than that of the PDB standard ($^{13}C/^{12}C$ PDB = 0.011237). Corrections are applied to the measured differences, including any zero enrichment in the capillary inlet system, valve mixing between sample and standard valves, and tailing of major onto minor peak signal.

Results and Recommendations

The results of our recent survey (9) are given in Table 2. The probability of a given orange juice sample being unadulterated may be determined from multiples of the standard deviation, as shown in Table 3. A sample with a value less negative than −22.1‰ should be classified as adulterated. Because of the range of values found for pure orange juices (−23.4‰ to −25.6‰), a statistical approach is necessary to interpret the results of the test.

The results of the collaborative study are compiled in Table 4. Regression lines of percent orange juice on $\delta^{13}C$ (range of values for all collaborators) demonstrate the good agreement among the collaborators and are shown in Figure 1. These lines were all significant ($P < 0.01$) with very high correlation coefficients, $-1.00 < r < -0.99$. All adulterated samples in the study

Table 2. $\delta^{13}C$ Values of orange juice and HFCS[a]

Sample	No. of samples	Mean, ‰	Range, % Low	Range, % High	SD	CV, %
Orange juice	42	−24.5	−25.6	−23.4	0.591	2.41
HFCS	4	−9.7	−9.8	−9.5	0.14	1.4

[a] See ref. 9 for individual values for 42 orange juice samples, including 3 domestic blends, 7 samples of varietal purity, and 4 foreign samples.

Table 3. Probability of $\delta^{13}C$ value of authentic orange juice sample being more negative than stated limit

Probability, %	Limit of $\delta^{13}C$, ‰
5 of 6 = 84.1	−23.9
43 of 44 = 97.72	−23.3
769 of 770 = 99.87	−22.7
24 999 of 25 000 = 99.997	−22.1

Table 4. $\delta^{13}C$ (‰ vs PDB) for collaborative samples

	Sample				
Coll.	A	B	C	D	E
1	−20.2	−13.2	−24.9	−15.5	−18.0
2	−21.3	−14.8	−25.9	−17.2	−19.2
3	−21.3	−14.5	−26.0	−16.7	−19.2
4	−20.7	−14.2	−24.7	−16.0	−19.0
5	−21.5	−15.2	−25.3	−16.6	−18.9
6	−21.5	−14.5	−26.1	−16.7	−18.8
7	−21.2	−14.5	−26.2	−16.4	−18.8
Mean	−21.1	−14.4	−25.6	−16.4	−18.8

(A, B, D, E) were properly classified, according to the highest probability limit set in Table 3.

From these results, the Associate Referee recommends that the $^{13}C/^{12}C$ mass spectrometric method be adopted official first action for detecting HFCS in orange juice.

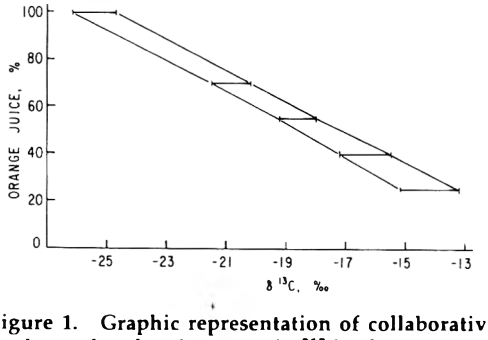


Figure 1. Graphic representation of collaborative study results, showing range in $\delta^{13}C$ values reported by all collaborators for orange juice with various levels of HFCS added.

Acknowledgments

The authors gratefully acknowledge the statistical counsel of John G. Phillips of this Center, and also the following collaborators: Odette Carro and Claude Hillaire-Marcel, Université du Québec à Montreal, Montreal, Québec, Canada; Robert Drimmie and Peter Fritz, University of Waterloo, Waterloo, Ontario, Canada; Juanita N. Gearing, University of Rhode Island, Kingston, RI; Patrick L. Parker, Coastal Science Laboratories, Inc., Port Aransas, TX; Richard M. Reeseman, Krueger Enterprises, Inc., Cambridge, MA; Bruce N. Smith, Brigham Young University, Provo, UT; Hubert Ziegler, Technischen Universitat München, Munich, GFR.

References

(1) Vandercook, C. E. (1977) *Food Chem.* **2,** 219–233
(2) Anonymous (1980) *Anal. Chem.* **52,** 1269A–1272A
(3) Petrus, D. R., & Vandercook, C. E. (1980) in *Citrus Quality and Nutrition*, S. Nagy and J. A. Attaway (Eds), ACS Symposium Series 143, American Chemical Society, Washington, DC, pp. 395–422
(4) Petrus, D. R., & Attaway, J. A. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 1317–1331
(5) Vandercook, C. E., Smolensky, D. C., Nakamura, L. K., & Price, R. L. (1976) *J. Food Sci.* **41,** 709–710
(6) Smolensky, D. C., & Vandercook, C. E. (1980) *J. Food Sci.* **45,** 1773–1780
(7) White, J. W., Jr, & Doner, L. W. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 746–750
(8) Doner, L. W., & Phillips, J. G. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 85–90
(9) Doner, L. W., & Bills, D. D. (1981) *J. Agric. Food Chem.* **29,** 803–804
(10) Nissenbaum, A., Lifshitz, A., & Stepak, Y. (1974) *Lebensm. Wiss. Technol.* **7,** 152–154

DAIRY PRODUCTS

Collaborative Evaluation of Rolling Ball Viscometer for Measuring Somatic Cells in Abnormal Milk

GARY H. RICHARDSON, RODNEY J. BROWN, RON CASE,[1] ROY E. GINN,[2] TED KASANDJIEFF,[3] and RICK C. NORTON[4]
Utah State University, Department of Nutrition and Food Sciences, Logan, UT 84322

The Ruakura rolling ball viscometer was evaluated in 3 laboratories along with currently approved instrumental methods for measuring somatic cells in milk and the Wisconsin mastitis test. Replacement of the Teepol reagent with Wisconsin mastitis test reagent in the rolling ball viscometer was also evaluated. Both repeatability and reproducibility were satisfactory for all methods evaluated. The instrumental methods each gave higher readings than the other 3 methods. Use of Wisconsin mastitis test reagent in the rolling ball viscometer improved both repeatability and reproducibility. Additional work on standardization is suggested to match rolling ball viscometer readings with those of the instrumental methods.

The Ruakura rolling ball viscometer (RBV) has been used in many New Zealand dairy plants to estimate somatic cell counts in milk (1, 2). Use of the device has apparent advantages over other methods which rely on the development of viscosity by addition of neutral detergents to milk (3). The scale of the instrument extends above that of the Wisconsin mastitis test (WMT) and it is not subject to orifice plugging problems. The ability to test 60 samples per hour and the relatively low instrument and operational costs suggest the RBV as a promising device for the dairy laboratory. A coefficient of variance (CV) for repeatability of 16–21% has been reported for the RBV (4). The CV for the WMT has been reported to vary from 3.5 to 64%, depending on the age of the sample before testing (5, 6). The RBV was previously evaluated in the United States (4); however, it has not been subjected to collaborative study. The AOAC General Referees for Dairy Products and for Microbiological Methods suggested a modified collaborative study of the RBV. Different samples were allowed in each collaborating laboratory because of rapid deterioration beyond 36 h (7). This paper summarizes the results of the study.

Experimental

Automation Engineering (Refrigeration Engineering Co., Ltd, 26 Great South Rd, Otahuhu, PO Box 12072, Aukland, New Zealand) supplied RBV instruments to each of the collaborating laboratories. Calibration and operational procedures were followed as outlined in the manufacturer's 1979 manual supplied with each instrument. Because the Teepol reagent used in New Zealand is neither biodegradable nor readily available in the United States, the WMT reagent was considered as a substitute. Both the standard Teepol reagent (RBV(T)) and the standardized WMT reagent diluted as in the WMT test (RBV(W)) were evaluated as reagents for the RBV tests. Procedures for the WMT were followed as outlined in *Standard Methods for the Examination of Dairy Products*, as updated (7, 8).

In addition to WMT, RBV(T) and RBV(W), each laboratory also used one instrumental method. Collaborator 1 used a Model MCC Coulter Counter (CC), Collaborator 2 used the DNA filter method (DNA), and Collaborator 3 used the Fossomatic method (FOSS). Each sample was tested in duplicate by each of the 3 common methods and by one instrumental method in each laboratory.

Each collaborating laboratory tested 50 fresh samples of raw milk which had been held for less than 36 h before testing. To obtain samples with a range of somatic cell counts, Collaborator 1 obtained mastitic milk from a single cow and blended it with normal milk samples. The other 2 collaborators found bulk milk samples over wide ranges of somatic cell counts.

Cell numbers were estimated from WMT results, using data of Thompson and Postle (9). A regression equation:

Contribution No. 2640 of the Utah Agricultural Experiment Station. Approved by the Director.

[1] Kraft Inc., 500 Pestigo St, Chicago, IL 60690.

[2] Dairy Quality Control Institute, 2353 Rice St, St. Paul, MN 55113.

[3] Dairyman's Cooperative Creamery Association, 400 S M St, Tulare, CA 93274.

[4] Kroger Co., 1212 State Ave, Cincinnati, OH 45204.

Mention of companies or products does not constitute endorsement by Utah State University over comparable products.

Received November 12, 1980. Accepted October 27, 1981.

0004-5756/82/6503-0611-05$01.00

Table 1. Separate analyses of variance for each of 3 methods used in all 3 laboratories

Source of var.	DF	Mean sq.	*F*-ratio	Alpha	Expected mean sq.
			RBV(T)		
Laboratory	2	2.022	16.43	0.0001	$\sigma_E^2 + n\sigma_S^2 + s\kappa_L^2$
Sample	63	0.123	41.81	0.0001	$\sigma_E^2 + n\sigma_S^2$ —
Error	66	0.003	—	—	σ_E^2 — —
Corrected total	131	—	—	—	— — —
			RBV(W)		
Laboratory	2	0.939	10.23	0.0001	$\sigma_E^2 + n\sigma_S^2 + sn\kappa_L^2$
Sample	63	0.092	67.75	0.0001	$\sigma_E^2 + n\sigma_S^2$ —
Error	66	0.001	—	—	σ_E^2 — —
Corrected total	131	—	—	—	— — —
			WMT		
Laboratory	2	1.329	35.06	0.0001	$\sigma_E^2 + n\sigma_S^2 + sn\kappa_L^2$
Sample	63	0.038	27.37	0.0001	$\sigma_E^2 + \sigma_S^2$ —
Error	66	0.001	—	—	σ_E^2 — —
Corrected total	131	—	—	—	— — —

Somatic cell count = (mm left in WMT tube − 5.88)/20 which had a correlation coefficient of 0.91 in the original study was used to make this transformation. Somatic cell count data for all 6 methods were converted to log values before further statistical analysis (10). The RBV(T) and RBV(W) methods are limited to the range of 2.0×10^5 to 3.0×10^6 counts/mL. The WMT method is restricted to the range from 6.0×10^3 to 1.2×10^6 cells/mL. For this reason, all samples which gave readings outside these limits were discarded, even if valid measurements were possible by the other methods. The data range for the study was thus set from 2.0×10^5 to 1.2×10^6 by the limits of the RBV(T), RBV(W), and WMT methods (10). The SAS statistical package was used for much of the statistical analysis (11).

Results and Discussion

Variability between samples was adequate for statistical testing even after extreme values were eliminated as described above (Tables 1 and 2). Standard deviation (SD) and CV values for repeatability of the 6 methods shown in Tables 1 and 2 were, respectively, RBV(T) 0.054, 0.94%; RBV(W) 0.037, 0.65%; WMT 0.037, 0.64%; CC 0.012, 0.20%; DNA 0.052, 0.85%; and FOSS 0.022, 0.38%. These same measures for reproducibility

Table 2. Separate analyses of variance for each of 3 methods used in one laboratory each

Source of var.	DF	Mean sq.	*F*-ratio	Alpha	Expected mean sq.
			CC		
Sample	26	0.115	769.1	0.0001	$\sigma_E^2 + \sigma_S^2$
Error	27	0.0002	—	—	σ_S^2 —
Corrected total	53	—	—	—	— —
			DNA		
Sample	10	0.025	9.5	0.0004	$\sigma_E^2 + \sigma_S^2$
Error	11	0.0027	—	—	σ_S^2 —
Corrected total	21	—	—	—	— —
			FOSS		
Sample	27	0.042	91.58	0.0001	$\sigma_E^2 + \sigma_S^2$
Error	28	0.0005	—	—	σ_E^2 —
Corrected total	55	—	—	—	— —

Table 3. Analysis of variance and Duncan's multiple range test comparing RBV(T), RBV(W), and WMT methods for estimation of somatic cell counts in all 3 laboratories

Analysis of Variance

Source of var.	DF	Mean sq.	*F*-ratio	Alpha	Expected mean sq.
Laboratory	2	4.084	20.2	0.0001	$\sigma_E^2 + t n \sigma_S^2 + t s n \kappa_L^2$
Method	2	0.803	31.5	0.0001	$\sigma_E^2 + n \sigma_{TS}^2 + l s n \kappa_T^2$
Laboratory–method interaction	4	0.103	4.0	0.0041	$\sigma_E^2 + n \sigma_{TS}^2 + s n \kappa_{LT}^2$
Sample	63	0.202	106.5	0.0001	$\sigma_E^2 + t n \sigma_S^2$ —
Method–sample interaction	126	0.026	13.5	0.0001	$\sigma_E^2 + n \sigma_{TS}^2$ —
Error	198	0.002	—	—	σ_E^2 — —
Corrected total	395	—	—	—	— — —

Duncan's Multiple Range Test

Method	Number of samples	Mean reading[a]	Groupings[b]
RBV(T)	132	5.801	a
WMT	132	5.793	a
RBV(W)	132	5.662	—

[a] Values are log of count or conversion to comparable numbers as explained in text.
[b] Methods with the same letter are not significantly different at the alpha = 0.05 level.

of the methods in Table 1 were RBV(T) 0.215, 3.70%; RBV(W) 0.144, 2.54%; and WMT 0.175, 3.03% (10). This experiment did not allow determination of SD and CV for reproducibility of the methods in Table 2. Even though repeatability was excellent for all 6 methods, the Coulter counter was most precise and the RBV(T) was least precise. Reproducibility of the 3 methods common to all 3 laboratories was also good. The replacement of Teepol reagent with WMT reagent in the RBV method improved both repeatability and reproducibility.

Comparison of the methods common to all 3 laboratories (Table 3) indicates that RBV(T) and

Table 4. Analysis of variance and Duncan's multiple range test comparing RBV(T), RBV(W), WMT, and CC methods for estimation of somatic cell counts

Analysis of Variance

Source of variation	DF	Mean sq.	*F*-ratio	Alpha	Expected mean sq.
Method	3	0.871	28.81	0.0001	$\sigma_E^2 + n \sigma_{TS}^2 + s n \kappa_T^2$
Sample	26	0.387	290.46	0.0001	$\sigma_E^2 + t n \sigma_S^2$ —
Method–sample interaction	78	0.030	22.70	0.0001	$\sigma_E^2 + n \sigma_{TS}^2$ —
Error	108	0.001	—	—	σ_E^2 — —
Corrected total	215	—	—	—	— — —

Duncan's Multiple Range Test

Method	Number of samples	Mean reading[a]	Groupings[b]
CC	54	6.108	—
RBV(T)	54	6.009	a
WMT	54	5.959	a
RBV(W)	54	5.804	—

[a] Values are log of count or conversion to comparable numbers as explained in text.
[b] Methods with the same letter are not significantly different at the alpha = 0.05 level.

Table 5. Analysis of variance and Duncan's multiple range test comparing RBV(T), RBV(W), WMT, and DNA methods for estimation of somatic cell counts

Analysis of Variance

Source of variation	DF	Mean sq.	*F*-ratio	Alpha	Expected mean sq.
Method	3	1.149	66.51	0.0001	$\sigma_E^2 + n\sigma_{TS}^2 + sn\kappa_T^2$
Sample	10	0.084	23.44	0.0001	$\sigma_E^2 + tn\gamma_S^2$ —
Method-sample interaction	30	0.017	4.85	0.0001	$\sigma_E^2 + n\sigma_{TS}^2$ —
Error	44	0.004	—	—	σ_E^2 — —
Corrected total	87	—	—	—	— — —

Duncan's Multiple Range Test

Method	Number of samples	Mean[a] reading	Groupings[b]
DNA	22	6.075	—
WMT	22	5.748	a
RBV(T)	22	5.710	a
RBV(W)	22	5.526	—

[a] Values are log of count or conversion to comparable numbers as explained in text.
[b] Methods with the same letter are not significantly different at the alpha = 0.05 level.

WMT gave results which are not significantly different from each other and which are greater than those for RBV(W). These conclusions were verified when data from individual laboratories were analyzed separately (Tables 4–6). In every case RBV(T) and WMT gave results with insignificant differences and RBV(W) gave lower readings. Each of the instrumental methods gave higher readings than RBV(T) and WMT (Tables 4–6). The limitations of test sample range which apply to the RBV(T), RBV(W), and WMT methods do not apply for any of the instrumental methods. The RBV(W) method could be calibrated to match the other methods by better selection of reference glycerol solution or readjustment of glycerol concentration. Concentration of reagent may have more effect than type or source of reagent (1).

All of the collaborators preferred their instrumental methods to the WMT or RBV. Nei-

Table 6. Analysis of variance and Duncan's multiple range test comparing RBV(T), RBV(W), WMT, and FOSS methods for estimation of somatic cell counts

Analysis of Variance

Source of variation	DF	Mean sq.	*F*-ratio	Alpha	Expected mean sq.
Method	3	0.130	12.71	0.0001	$\sigma_E^2 + n\sigma_{TS}^2 + sn\kappa_T^2$
Sample	27	0.212	196.69	0.0001	$\sigma_E^2 + tn\sigma_S^2$ —
Method-sample interaction	81	0.010	9.52	0.0001	$\sigma_E^2 + n\sigma_{TS}^2$ —
Error	112	0.001	—	—	σ_E^2 — —
Corrected total	223	—	—	—	— — —

Duncan's Multiple Range Test

Method	Number of samples	Mean reading[a]	Groupings[b]
FOSS	56	5.694	—
WMT	56	5.651	a
RBV(T)	56	5.636	a
RBV(W)	56	5.578	—

[a] Values are log of count or conversion to comparable numbers as explained in text.
[b] Methods with the same letter are not significantly different at the alpha = 0.05 level.

ther of these methods would be appropriate in applications where reduction to 10^6 cells/mL is monitored (12). The RBV and WMT methods did correlate well with instrumental methods within the range limitations. High somatic cell count samples plugged the glass tube of the RBV and cleaning between samples reduced the rate of this method to 40–80 samples per hour. Duirs and Cox (1) have indicated that a throughput of up to 300 samples per day could make the RBV suitable for use in small laboratories. The CC and FOSS units used in this study now operate at over 210 samples per hour.

Recommendations

Due to its reproducibility when compared with WMT and RBV(T) and its excellent repeatability, it is suggested that the RBV(W) method be subjected to additional studies for refinement of the standardization and operational procedures, and then be considered for official action. The use of WMT reagent in the RBV is recommended because of its availability and its biodegradability as well as performance at least equal to that of the Teepol reagent. The standardization procedure could be adjusted to match RBV(W) results with those of WMT or RBV(T) by modifying glycerol concentration.

Acknowledgments

We were saddened by the death of Ted Kasandjieff who had volunteered to be a collaborator but became ill before the project was initiated. We are indebted to Colin R. Dillicar, Manager of Automation Engineering, for donating the rolling ball viscometers for this study. We also appreciate the donation of WMT reagent by Z. Doyle Roundy. Statistical assistance by Donald V. Sisson and David L. Turner is also appreciated.

References

(1) Duirs, G. F., & Cox, R. N. (1977) *N. Z. J. Dairy Sci. Technol.* **12**, 107–112
(2) Milne, J. R., & Smyth, R. (1976) *N. Z. J. Dairy Sci. Technol.* **11**, 21–23
(3) Fell, L. R., Whittlestone, W. G., & DeLangen, H. (1971) *J. Milk Food Technol.* **34**, 82–84
(4) Leonard, R. O., & Gurtle, G. (1975) *Calif. Agric.* July, 8–10
(5) Funk, C. D., Schultz, L. H., & Barr, G. R. (1967) *J. Dairy Sci.* **50**, 47–52
(6) Marchall, R. T., & Brechbuhler, J. D. (1977) *J. Food Prot.* **40**, 195
(7) Marth, E. H. (Ed.) (1978) *Standard Methods for the Examination of Dairy Products*, 14th Ed., APHA, Washington, DC, Secs 8.33, 8.42, B11.11, and B11.13
(8) Richardson, G. H., et al. (1980) *J. Food Prot.* **43**, 324–328
(9) Thompson, D. I., & Postle, D. S. (1964) *J. Milk Food Technol.* **27**, 271–275
(10) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA
(11) SAS Institute (1979) *SAS User's Guide*, Raleigh, NC
(12) Aly, E. A., Andrews, A. T., & Cheeseman, G. C. (1980) *J. Dairy Res.* **47**, 393–400

Chemiluminescence Detection of Nitrite in Nonfat Dried Milk Powders

ROBERT C. DOERR, ROBERT A. GATES, and WALTER FIDDLER
U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118

Determination of nitrite in nonfat dry milk powders by chemiluminescence detection was compared with a colorimetric method specifying Griess reagents. The chemiluminescent technique requires no sample preparation, is free from apparent interferences, and is sensitive to 25 ppb nitrite. Statistical analysis shows no difference at $P = 0.05$ between the colorimetric and chemiluminescent methods, based on the analysis of 16 commercial nonfat dry milk samples.

Nitrite in nonfat dry milk (NFDM) powder results from nitrogen oxides generated by the direct-fired dryers now commonly used in the United States. Recent reports (1, 2) of the presence of dimethylnitrosamine in NFDM have placed new importance on the determination of nitrite because of its role as a precursor in nitrosamine formation.

Nitrite in milk is generally determined colorimetrically using some form of Griess reaction, which is the basis of the method proposed by the International Organization for Standardization (ISO) in 1979 (3). However, the colorimetric method requires considerable sample preparation and is subject to error resulting from solution turbidity, color caused by pigment formation, and naturally occurring reductants. A chemiluminescence detection (CLD) method, unaffected by these problems, has been reported by Walters et al. (4) for nitrite analysis in a simulated food matrix. Cox (5), using sodium iodide as a reductant, determined nitrite in water solutions by CLD. Dahn and Loewe (6) studied the quantitative reduction of nitrite by ascorbate and reported a 92% conversion to nitric oxide at pH 5. Doerr et al. (7) used sodium ascorbate as a reductant to measure nitrite in cured meat slurries by CLD. The purpose of the present study is to determine whether the CLD method is suitable for measuring low levels of nitrite in NFDM.

Experimental

Milk

Two and one-half g of commercial NFDM powder was reconstituted with double deionized water to 25 mL. Two drops of Dow Corning Antifoam A silicone defoamer were added to prevent excessive foaming during mixing.

Reagents

All chemicals were reagent grade or highest purity available and were used without further purification.

Double deionized water was prepared by passing tap water through a charcoal filter, a mixed bed ion exchange resin, a Barnstead standard ion exchange cartridge, and, finally, a Barnstead organic removal column.

Chemiluminescence Method (7)

Two mL of reconstituted milk was added to the reaction flask containing ca 56 mg solid sodium ascorbate, 44 mg solid tartaric acid, and 20 μL silicone antifoam agent and stirred vigorously. The evolved nitric oxide was injected into a thermal energy analyzer (Model 502, Thermo Electron Corp., Waltham, MA) by means of a Carle No. 5518 6-port minivalve (Carle Instruments, Inc., Anaheim, CA) as described elsewhere (7). Peak areas were measured by the integrator supplied with the thermal energy analyzer. Sodium nitrite concentrations were calculated by comparing sample peak areas with those obtained from aqueous standard solutions of sodium nitrite at similar concentrations. Standards were measured several times a day to assure good repeatability. Solvent and reagent blanks were analyzed daily and contained insignificant levels of nitrite. However, all sample calculations were corrected for these low values. A minimum of 3 injections for each sample was used to determine the mean injection value.

Colorimetric Method

Solutions.—(a) *Carrez I.*—17.2 g potassium ferrocyanide ($K_4Fe(CN)_6.3H_2O$) diluted to 100 mL with water.

(b) *Carrez II.*—53.5 g zinc sulfate ($ZnSO_4.7H_2O$) diluted to 100 mL with water.

Received September 15, 1981. Accepted November 20, 1981.

Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

0004-5756/82/6503-0616-03$01.00

Table 1. Nitrite in commercial nonfat dry milk powders

	Chemiluminescence			Colorimetric (Griess)		
Sample	$NaNO_2$,[a] ppb	SD	CV, %	$NaNO_2$,[a] ppb	SD	CV, %
1	35	7	20	350	0	0
2	425	120	28	720	170	24
3	990	127	13	1400	148	11
4	1090	191	17	1670	226	13
5	1110	85	7.6	1280	0	0
6	1510	148	9.8	1510	7	0.5
7	1630	57	3.5	1780	7	4.0
8	2160	49	2.3	2350	198	8.4
9	2550	106	4.1	2370	49	2.1
10	2580	7	0.3	2800	64	2.3
11	2610	325	12	3120	403	13
12	3480	99	2.8	3440	290	8.4
13	3710	516	14	3740	134	3.6
14	3910	361	9.2	4200	311	7.4
15	4280	84	2.0	4230	156	3.7
16	8670	955	11	7750	290	3.7

[a] Average of duplicate analyses.

(c) *Buffer.*—5 mL concentrated HCl added to 50 mL solution containing 10 mL concentrated NH_4OH and 40 mL water. Resulting solution was diluted to 100 mL with water.

(d) *Griess reagent.*—0.133 g *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NED) and 0.333 g sulfanilamide dissolved in 100 mL 15% acetic acid solution.

Procedure.—Twenty mL reconstituted milk was added to a 50 mL centrifuge tube; 4.0 mL Carrez II, 4.0 mL Carrez I, and 3.0 mL buffer were added in sequence. The solution was stirred thoroughly after each addition and left to stand 5 min after the final addition. The solution was centrifuged at 15 000 rpm for 15–20 min, and the supernatant liquid was filtered through Whatman No. 50 paper which had been previously washed for 30 min with deionized water to remove residual nitrite and then dried in a 115°C oven for 1.5 h. One mL Griess reagent was added to 5 mL filtrate, and the solution was diluted to 10 mL. After a color developing time of 20 min, the absorbance was measured at 515 nm with a Sargent Welch Model 6-550 UV/visible spectrophotometer. The nitrite concentration was calculated by comparing the final absorbance to a calibration curve. The final absorbance was determined by measuring absorbance of the sample plus Griess reagent vs Griess alone and subtracting from this value the absorbance of undiazotized milk. It was necessary to make this correction because the milk samples had a slight color that would be measured erroneously as nitrite.

The colorimetric procedure used in this study is similar to the ISO method for nitrite in dried milk except that we add a centrifugation step before filtration, and our final sample concentration is approximately 3 times greater than that recommended. The higher concentration was necessary to measure nitrite at the low ppb level. As a result, the volume of precipitate formed represented a significant part of the total volume, thereby making it necessary to measure the volume of supernatant liquid after centrifugation in order to calculate the final nitrite concentration.

Results and Discussion

Recovery of Added Nitrite

Milk powder dried by indirect heat and containing low apparent nitrite was spiked with sodium nitrite to yield a final concentration of 250, 2000, and 10 000 ppb. Recoveries of duplicate analyses were 75% at the 250 ppb level, 81% at the 2000 ppb level, and 91% at the 10 000 ppb level for CLD, and 82 and 88% at the 2000 and 10 000 ppb levels, respectively, for the Griess method.

Linearity, Sensitivity, Repeatability

The nitric oxide response was linear over a range equivalent to 25–20 000 ppb sodium nitrite in NFDM powder. The theoretical detection limit for a 10 mL sample and assuming a 2:1 signal-to-noise ratio was 2.5 ppb in the reconstituted milk. From a practical point of view, 25 ppb

Table 2. Analysis of variance for CLD vs colorimetric methods for nitrite

Source	DF	SS	MS	F	Signif. level
Sample	15	22.4×10^7	14.9×10^6	220	***
Method	1	24.3×10^4	24.3×10^4	3.58	NS[a]
Sample × method	15	18.1×10^5	12.1×10^4	1.78	NS
Error	32	21.7×10^5	67.7×10^3	—	—
Total	63	22.8×10^7	—	—	—

*** $P < 0.001$.
[a] NS = not significant.

sodium nitrite was considered the minimum level of reliable measurement, based on 75% average recovery from spiked samples containing low levels of normally incurred nitrite.

For the determination of instrument repeatability, 16 determinations (n = 16) of 500 ppb $NaNO_2$ standard solutions were made over a period of several weeks. The standard deviation (SD) and coefficient of variation (CV) were 23 ppb and 4.6%, respectively. During the same period, a commercial milk sample was analyzed 5 times and was determined to contain 600 ± 16 ppb sodium nitrite (CV 2.7%).

Nitrite Stability

Great care should be exercised when using very dilute standard nitrite solutions. We found that, even for samples stored under ideal conditions (brown bottle, cool environment), sudden loss of nitrite can occur without apparent reason. In several cases, after days of remaining at a constant value, a solution of 500 ppb sodium nitrite decreased in concentration by 90% overnight. Therefore, it is recommended that standards, particularly very low concentrations, be checked regularly to ensure that no loss has occurred.

Comparative Determination of Nitrite

Table 1 lists the mean results of duplicate nitrite analyses, uncorrected for percent recovery, of 16 commercial nonfat dry milk samples by the CLD and colorimetric methods. A comparison of the mean differences and coefficients of variation by a 2-tailed paired t-test indicates no significant differences at the P = 0.05 level.

Table 2 shows the results of analysis of variance of the comparison of the CLD and Greiss nitrite analyses. The data show that over a wide range of sample levels there is no significant (P = 0.05) difference between the 2 methods. In addition, the interaction between the range of sample levels and the different methods shows no significant effect statistically.

Since significant differences were not apparent in the statistical tests (P = 0.05), it was concluded that the results of the 2 methods were equivalent.

These results indicate that inorganic nitrite can be determined reliably in powdered milk by CLD with no sample preparation at a minimum detectable level of 25 ppb that is unattainable by other methods.

Acknowledgment

The authors thank Jay B. Fox, Jr, for his advice and assistance in carrying out this study and Thomas Fazio and Donald Havery, FDA. Bureau of Foods, for supplying the nonfat dry milk powder samples.

References

(1) Libbey, L. M., Scanlan, R. A., & Barbour, J. F. (1980) *Food Cosmet. Toxicol.* **18**, 459-461
(2) Lakritz, L., & Pensabene, J. W. (1981) *J. Dairy Sci.* **64**, 371-374
(3) Draft Proposal ISO/DP No. 6736 (1979) (Derived from ISO No. 4099), ISO, Geneva, Switzerland
(4) Walters, C. L. Downes, M. J., Hart, R. J., Perse, S., & Smith, P. L. R. (1978) *Z. Lebensm. Unters. Forsch.* **167**, 229-232
(5) Cox, R. D. (1980) *Anal. Chem.* **52**, 322-325
(6) Dahn, H., & Loewe, L. (1960) *Helv. Chim. Acta* **43**, 287-333
(7) Doerr, R. C., Fox, J. B., Lakritz, L., & Fiddler, W. (1981) *Anal. Chem.* 53, 381-384

VITAMINS AND OTHER NUTRIENTS

Simultaneous Determination of Vitamins A and D in Dosage Forms by High Pressure Liquid Chromatography

MARIA INES R. M. SANTORO, JOÃO F. MAGALHÃES, and ERIKA R. M. HACKMANN

Universidade de São Paulo, Faculdade de Ciências Farmacêuticas, Caixa Postal 30.786, São Paulo, Brasil

Vitamins A and D were determined simultaneously in oily solutions, ointments, and elixirs, but only vitamin A could be determined in capsules. Samples were saponified with KOH in isopropanol-water, using hydroquinone as antioxidant, and extracted with ether-petroleum ether (1 + 1). After evaporation of solvent, residues were dissolved in isopropanol. Vitamins in these solutions were determined by reverse phase high pressure liquid chromatography, using methanol-water as mobile phase and detection at 254 nm. The reproducibility, using external standards, was 1.6-2.5% and 1.2-3.8% for vitamins A and D, respectively.

The classic and official methods for determination of vitamins A and D are generally lengthy because they require many steps to eliminate interfering substances. The problem increases with the complexity of the samples, especially for the determination of vitamin D in the presence of vitamin A.

In the past few years, high pressure liquid chromatography (HPLC) has been a useful technique for analyzing many compounds present in mixtures and, among them, the fat-soluble vitamins. An extensive review of analysis of drugs and their metabolites by HPLC was published by Wheals and Jane (1).

A collaborative study was carried out in which vitamin D in concentrates and in the presence of vitamin A was determined. Two HPLC methods were developed (2–6) and compared with the official (chemical) AOAC method for vitamin D in concentrates.

Mackay et al. (7) described an HPLC method for determination of vitamin D_2 in multivitamin tablets. Other fat-soluble vitamins such as vitamins A (acetate), A (alcohol), and E present in the samples did not interfere with the assay. Williams et al. (8) studied a method that permits the determination of fat-soluble vitamins and their esters, and Krol et al. (9) applied a chromatographic system to the separation of vitamins A (acetate), A (palmitate), A (aldehyde), A (alcohol), E, and D. The separation of vitamins A, D, and E was also reported by Vermont et al. (10) and Bombaugh (11). Vitamin D_3 and its isomers in D_3 resins and in mixtures with vitamin A (acetate) were separated and quantitatively determined by HPLC by Steuerle (12). Osadca and Araujo (13) described a method for determining the presence of vitamin D_2 and/or vitamin D_3 in various preparations containing vitamins A and E. Simultaneous determination of vitamins A, D, and E in multivitamin tablets was reported by Eriksson et al. (14) and Barnett and Frick (15).

Because of the importance of the studies being carried out in this field, we developed a method that has sufficient selectivity, precision, and accuracy to be applied to the determination of vitamins A and D contained in various commercially available dosage forms. The efficiency of an HPLC method for the analysis of fat-soluble vitamins, in most cases, depends on the sample preparation. Three important factors must be considered: the effective extraction of the vitamins; the elimination, as far as possible, of interfering substances; and the conservation of the integrity of the vitamins. Considering these factors, we tested the extraction procedures indicated by Williams et al. (8) and Barnett and Frick (15). In these procedures, samples are not saponified. Known amounts of vitamin A were added to placebo capsules, extracted, and analyzed by the ultraviolet spectrophotometric method and colorimetric method with Carr-Price reagent.

In addition, placebos containing known amounts of vitamin A or D were saponified, extracted with ether-petroleum ether (1 + 1), and analyzed by the methods mentioned above.

METHOD

Reagents

(a) *Methanol.*—Spectrograde (Aldrich).
(b) *HPLC water.*—Baker.
(c) *Isopropanol.*—Spectrograde (Merck).
(d) *Standards.*—Vitamin A (acetate) standard

0004-5756/82/6503-0619-05$01.00

Table 1. Data for recovery of vitamin A (acetate) added to capsules [a]

	Ultraviolet		Colorimetric		
IU added	Found, IU	Recovery, %	Found, IU	Recovery, %	Extn ref.
25 000	15 152	60.6	17 000	68.0	(8)
25 000	16 757	67.0	18 382	73.5	(8)
50 000	27 500	55.0	36 600	73.2	(8)
25 000	6307	25.2	6175	24.7	(15)
50 000	22 230	44.4	24 665	49.3	(15)
50 000	31 690	63.3	31 430	62.8	(15)

[a] Samples analyzed without saponification. Final concentration 10 IU/mL.

solution: containing 240 or 500 IU/mL isopropanol. Vitamin D_2 or D_3 standard solution: containing 80 IU/mL isopropanol. Keep standards in the dark at 4°C and prepare fresh each day.

Apparatus

(a) *High pressure liquid chromatograph.*—Varian Model 5020, equipped with 254 nm UV detector, manual sample injection valve with 10 μL loop, Model 261 recorder, and Model CDS-111 integrator.

(b) *Column.*—Stainless steel, 30 cm × 4 mm id, containing MicroPak CH-10.

Samples

Samples, simulating commercially available preparations, were composed of the following:

(a) *Oily solution.*—25 000 IU vitamin A (acetate) and 4000 IU vitamin D_3/mL

(b) *Ointment.*—5000 IU vitamin A (palmitate) and 800 IU vitamin D_3/g.

(c) *Elixir.*—3000 IU vitamin A (palmitate), 1000 IU vitamin D_2, 3 mg thiamine hydrochloride, 3 mg riboflavin phosphate, 2 mg pyridoxine hydrochloride, 1 mg nicotinamide, and 0.5 mg panthenol/5 mL.

(d) *Capsule.*—25 000 IU vitamin A (acetate), 4000 IU vitamin D_2, 10 mg thiamine mononitrate, 5 mg riboflavin phosphate, 5 mg pyridoxine hydrochloride, 100 mg ascorbic acid, 5 mg α-tocopherol acetate, 2 mg menadione, 50 mg niacinamide, and 10 mg calcium pantothenate/500 mg.

Keep samples in the dark at 4°C and in amber glassware. Carry out experiments in subdued light.

Saponification and Extraction

An amount of sample containing 25 000 IU vitamin A and 4000 IU vitamin D (oily solution, ointment, and capsule) or 12 000 IU vitamin A and 4000 IU vitamin D (elixir) was refluxed 30 min on a water bath with 100 mL isopropanol and 10 mL 50% w/v aqueous KOH in the presence of 500 mg hydroquinone antioxidant. Gelatin-coated vitamins from capsules were refluxed 15 min first with hydroquinone, KOH, and 20 mL water to facilitate the dissolution of gelatin. Then the isopropanol was added.

After cooling, 100 mL of a saturated solution of NaCl was added and the mixture was extracted with 100 and 50 mL ether–petroleum ether (1 + 1). The organic extracts were washed with 25 mL of a saturated solution of NaCl, mixed with 10 g anhydrous Na_2SO_4, and evaporated in a rotatory evaporator to dryness. The residue was dissolved in 50 mL isopropanol for analysis. The same conditions of saponification and extraction were repeated for a placebo of each sample and for the isolated standards of each vitamin.

High Pressure Liquid Chromatography

The final solution of samples, standards, and placebos in isopropanol were injected into the liquid chromatograph and eluted with methanol–water (95 + 5) at a gradient from 1.0 mL to 2.0 mL/min in 5 min. All analyses were carried out at ambient temperature; 10 injections were made for each sample. Standards and placebos were injected 3 times for each analysis. Concentrations of vitamins were obtained by comparing peak areas of samples with the average of peak areas of standards.

Results and Discussion

Extraction procedures without saponification did not provide quantitative results, as shown in Table 1. Probably the extraction of vitamin A under the experimental conditions was not complete; even after the eighth extraction, the

Table 2. Data for recovery of vitamins A (acetate or palmitate) and D (D_2 or D_3) added separately to placebos[a]

	IU added to placebos		Vitamin A				Vitamin D			
			Ultraviolet		Colorimetric		Ultraviolet		Colorimetric	
Sample	A	D	Found, IU	Recovery, %	Found, IU	Recovery, %	Found, IU	Recovery, %	Found, IU	Recovery, %
Oil soln	5000	2000	5000	100.0	5000	100.0	—[b]		2063	103.1
	5000		4737	94.7	5000	100.0				
Ointment	5000	500 000	5490	109.8	5026	100.5	—[b]		473 250	94.6
	25 000		24 382	97.5	25 000	100.0				
Elixir	10 000		9976	99.7	10 401	104.0				
	12 000		12 867	107.2	11 964	99.7				
Capsule	25 000	500 000	23 525	94.1	25 555	102.2	487 250	97.4	486 600	97.3
		1000 000					898 100	89.1	974 000	97.4

[a] Samples analyzed with saponification. Final concentrations: A = 10 IU/mL; D = 400 IU/mL.
[b] It was not possible to calculate concentrations because of interference by other substances in the formulation.

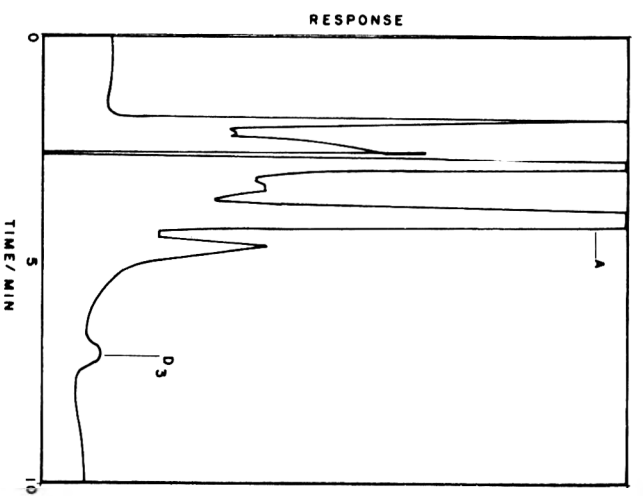


Figure 1. Liquid chromatogram of 10 μL of vitamins A acetate (5 IU) and D_3 (0.8 IU) after saponification and extraction.

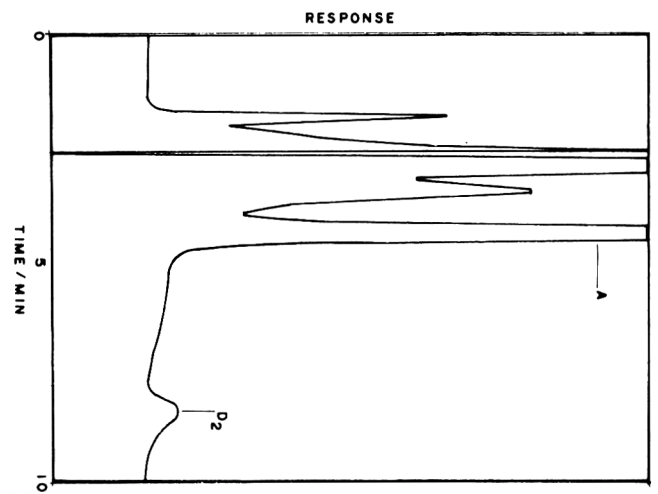


Figure 2. Liquid chromatogram of 10 μL of vitamins A palmitate (2.4 IU) and D_2 (0.8 IU) after saponification and extraction.

Table 3. Vitamins A and D found by proposed HPLC method in simulated commercially available preparations

Preparation	Declared, IU	Found, IU (CV, %)	Retention, min
1. Oily soln:			
A (acetate), IU/mL	25 000	25 215 (2.5)	4.05
D_3, IU/mL	4000	3989 (1.9)	7.12
2. Ointment:			
A (acetate), IU/g	5000	5011 (2.5)	4.05
D_3, IU/g	800	798 (1.2)	7.15
3. Elixir:			
A(palmitate), IU/mL	3000	3036 (1.6)	4.04
D_2, IU/mL	1000	1006 (3.8)	8.45
4. Capsule:			
A (acetate), IU/cap.	25 000	24 974 (2.6)	4.04
D_2, IU/cap.	4000	—	—

qualitative reaction of the organic extracts with Carr-Price reagent was still positive.

Recovery of vitamins A and D added separately to placebos was quantitative when saponification and extraction were included. Results are presented in Table 2. Based on these results, extraction including saponification was chosen for the preparation of samples to be analyzed by HPLC.

A good separation of vitamins A and D in dosage forms was obtained by using reverse phase HPLC. Figures 1 and 2 illustrate the chromatograms of oily solution and elixir, respectively. Vitamin concentrations in the 4 simulated commercial preparations assayed and their retention times are shown in Table 3. The coefficients of variation range from 1.6 to 2.5% for vitamin A, and 1.2 to 3.8% for vitamin D. These results demonstrate that the method is adequate for quantitative and qualitative determination of vitamins A and D in oily solutions, ointments, and elixirs.

The results for vitamin D refer to the total content of this vitamin in the pharmaceutical preparations studied. Under the experimental chromatographic conditions tested, we could not separate vitamin D, previtamin D, and other isomers that could be formed during the process. For this reason, standards and samples were always treated exactly the same.

For gelatin-coated vitamins A and D in capsules, vitamin D was not separated from interferences of substances resulting from the hydrolysis of gelatin. Nevertheless, we could quantitatively analyze vitamin A in the same sample.

Received June 29, 1981. Accepted November 14, 1981.

Placebos, samples, and standards were treated under the same conditions so we could observe interference due to the components of the formulations in the chromatograms in the regions corresponding to the vitamin peaks. Peaks that appeared were in regions that did not interfere with those of the vitamins.

Recovery data for vitamins added to placebos, presented in Table 2, show the efficiency of extraction with saponification. Results of the chromatographic analysis indicate that this method can be applied to various pharmaceutical preparations containing vitamins A and D, although it cannot identify the presence of previtamin D or other vitamin D isomers.

REFERENCES

(1) Wheals, B. B., & Jane, I. (1977) *Analyst* **102,** 625–644
(2) DeVries, E. J., Zeeman, J., Esser, R. J. E., Borsje, B., & Mulder, F. J. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 129–135
(3) DeVries, E. J., Zeeman, J., Esser, R. J. E., Borsje, B., & Mulder, F. J. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 1285–1291
(4) Hofsass, H., Alicino, N. J., Hirsch, A. L., Ameika, L., & Smith, L. D. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 735–745
(5) Hofsass, H., Grant, A., Alicino, N. J., & Greenbaum, S. B. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 251–260
(6) Mulder, F. J., DeVries, E. J., & Borsje, B. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 1031–1040
(7) Mackay, C., Tillman, J., & Burns, D. T. (1979) *Analyst* **104,** 626–636
(8) Williams, R. C., Schmit, J. A., & Henry, R. A. (1972) *J. Chromatogr. Sci.* **10,** 494–501
(9) Krol, G. J., Mannan, C. A., Gemmil, F. Q., Hicks, G. E., & Kho, B. T. (1972) *J. Chromatogr.* **74,** 43–49
(10) Vermort, J., Deleuil, M., DeVries, A. J. & Guil-

lemin, C. L. (1975) *Anal. Chem.* **47,** 1329–1337
(11) Bombaugh, K. J. (1975) *J. Chromatogr.* **107,** 201–206
(12) Steuerle, H. (1975) *J. Chromatogr.* **115,** 447–453
(13) Osadca, M., & Araujo, M. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 993–997
(14) Eriksson, M., Eriksson, T., & Sörensen, B. (1978) *Acta Pharm. Suec.* **15,** 274–281
(15) Barnett, S. A., & Frick, L. W. (1979) *Anal. Chem.* **51,** 641–645

High Performance Liquid Chromatographic Determination of Vitamin D in Fortified Milks, Margarine, and Infant Formulas

JAMES N. THOMPSON, GEORGE HATINA, WILLIAM B. MAXWELL, and SUZANNE DUVAL

Health and Welfare Canada, Nutrition Research Laboratories, Food Directorate, Tunney's Pasture, Ottawa, Ontario, Canada K1A 0L2

Fortified milks were saponified overnight at room temperature with 1% ethanolic pyrogallol and KOH. The digest was extracted with hexane after adding water and ethanol, and the extract was washed consecutively with 5% KOH, water, and 55% aqueous ethanol to remove polar lipids. After evaporation, the residue was first chromatographed on a column of 5 μm silica. A fraction containing vitamin D was collected, evaporated, and rechromatographed on a reverse phase column for the separation and quantitation of vitamins D_2 and D_3. Recovery was 96-99% and the coefficient of variation was 3% (8 replicates). Infant formula was diluted and then saponified and extracted as in the analysis of milk. Margarine was saponified by shaking overnight with 1% ethanolic pyrogallol and 80% KOH. Water and ethanol were added to the digest before extraction. Extracts from formula and margarine were chromatographed as milk except, before HPLC, the extract was dissolved in isopropanol-hexane (1 + 99) and passed through 5 cm alumina in a Pasteur pipet, and the concentration of isopropanol in the first high performance liquid chromatographic (HPLC) solvent system was halved to improve the separation of vitamin D from other absorbing lipids. Usually several peaks were obtained during the final HPLC analysis, and the identification of vitamins D_2 and D_3 was less certain than in the analysis of milk. The coefficients of variation for formula and margarine were 6% (5 replicates) and 9% (6 replicates), respectively.

Of all the vitamins added to food, probably none should arouse more concern or merit more vigilance than vitamin D added to milk. It is at the same time the most beneficial of vitamin additions, preventing infantile rickets and adult osteomalacia, and potentially the most dangerous because of the possible toxicity of small excesses.

Although the determination of vitamin D has been attempted with a variety of chromatographic and colorimetric techniques (1, 2), methods suitable for routine use were developed only after the introduction of high performance liquid chromatography (HPLC). This technique has been used to measure vitamin D in various fortified and unfortified materials including feed and food supplements (3-5), cod liver oil (6), fish products (7), commercial resins (8), and foods and feeds (9-11).

We earlier published a routine procedure for milk in which samples were saponified and the unsaponifiable lipid was purified in a time-consuming cleanup chromatography on a lipophilic gel, hydroxyalkoxypropyl Sephadex (HAPS). A portion of the eluate containing vitamin D was then subjected to HPLC on a silica column (12).

Henderson and Wickroski (13) later described a method in which the unsaponifiable lipid was chromatographed on a column of alumina before HPLC in a reverse phase system. More recently (14), it has been claimed that the cleanup chromatography on alumina can be omitted. However, we have been unable to confirm that vitamin D can be measured directly in unsaponifiable lipids from milk by reverse phase HPLC.

Adachi and Kobayashi (15) used several steps, including column chromatography, thin layer chromatography, and HPLC, in the measurement of natural levels of vitamin D in milk but this procedure is too complicated for routine analysis. Cohen and Wakeford (16) measured vitamin D relatively rapidly in nonfat dried milk by using HPLC but their method was not applicable to whole or partially skimmed milk.

We modified several steps in our original procedure (12), and the HAPS column has been eliminated. The method described below can be used routinely for the analysis of milk, and modifications are described for the analysis of other fortified foods such as margarine and infant formula.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Two isocratic systems including 2 pumps (Model 6000A, Waters Associates Inc.), 2 loop injectors (U6K, Waters Associates Inc.), 2 pen recorders, two 254 nm UV

Received September 21, 1981. Accepted December 8, 1981.
This paper was presented in part at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.

0004-5756/82/6503-0624-08$01.00

detectors (Model 440, Waters Associates Inc.), and one calculating integrator (Minigrator, Spectra-Physics, Santa Clara, CA 95051). Although it is possible to perform the 2 HPLC steps with one HPLC system, it is more convenient and less time-consuming to use 2 independent systems.

(b) *Silica column for HPLC.*—Column 4.6 × 150 mm of 5 μm spherical silica (Supelcosil LC-Si; Supelco).

(c) *Reverse phase column for HPLC.*—Column 4.6 × 250 mm of 10 μm spherical packing (Spherisorb 10 ODS; Spectra-Physics) or 8 mm C18, 5 μm Radial-Pak (Waters Associates Inc.).

(d) *Solvents.*—Reflux absolute alcohol 8 h with silver nitrate (5 g/L) and KOH (10 g/L) and distill. Use glass-distilled hexane for extractions and HPLC grade hexane, cyclohexane, acetonitrile, methanol, and isopropanol for chromatography.

(e) *Alumina.*—Immerse 200 g neutral alumina (AG7, Bio-Rad Laboratories) in hexane in mortar, add 10 mL water, and grind gently until uniform. Store deactivated alumina under hexane in airtight 500 mL screw-cap bottle. Test activity of alumina and recovery of vitamin D as follows: Add slurry of alumina in hexane, using Pasteur capillary pipet fitted with rubber teat, to 3 Pasteur pipets (230 mm) clamped vertically and plugged with glass wool. Add sufficient alumina to produce a column ca 5 cm high. Pass through each column 5 mL 1% isopropanol in hexane and discard eluate. Add 1 mL standard solution of vitamin D in hexane (ca 40 IU/mL; see below) to each column followed by 12 mL 1% isopropanol in hexane and collect the eluates in 15 mL centrifuge tubes. Place second set of tubes beneath columns and elute again with 12 mL 1% isopropanol in hexane. Evaporate solvent in tubes under nitrogen, transfer with hexane to 1 mL Reactivials (Pierce Chemical Co., Rockford, IL) and redissolve in 1 mL (first set) or 0.1 mL (second set) hexane. Inject 80 μL of each solution and the standard into HPLC silica column (see below) and measure vitamin D concentration. Vitamin should be recovered completely in first eluates and should not be detectable in second. If vitamin D is detected in second set of tubes, add more water to alumina and repeat test.

(f) *Solvent systems for HPLC.*—For silica column: 0.5% (milk analysis) or 0.25% (margarine and infant formula analysis) isopropanol in cyclohexane–hexane (1 + 1). For reverse phase systems: methanol–acetonitrile (1 + 9) with Spherisorb 10 ODS or methanol with Waters Radial-Pak A.

(g) *Standard solutions of vitamins D_2 and D_3.*—Store crystalline vitamin D_2 and vitamin D_3 at −10°C. Prepare solutions of each vitamin in methanol containing ca 10 μg vitamin D/mL, read in spectrophotometer at 265 nm, and calculate exact concentration in IU from:

$$\text{Concentration (IU/mL)} = \frac{100 \times \text{absorbance}}{0.120}$$

Dilute 10-fold in reverse phase solvent system to prepare solutions containing ca 40 IU/mL. Prepare also solution containing mixture of 40 IU vitamin D_2 and 40 IU vitamin D_3/mL. Store at −10°C. Before use, equilibrate flask ≥30 min at room temperature. Store at −10°C immediately after use. Prepare solutions of similar concentration in hexane and in isopropanol–cyclohexane–hexane to test alumina cleanup columns and to measure retention times on HPLC silica column.

Saponification and Extraction of Milk

Place 15 mL milk in 50 mL stoppered Erlenmeyer flask. Add 15 mL 1% ethanolic pyrogallol, and cool the flask in crushed ice. Add 6 g solid KOH and quickly replace stopper. Swirl until KOH is completely dissolved, remove from ice-bath, and let stand 18 h in darkness at room temperature. Transfer to 125 mL separatory funnel, using 15 mL water and 5 mL ethanol, followed by 45 mL hexane to rinse flask. Shake vigorously 1 min and let separate. If necessary, add a few drops of ethanol to induce separation. Transfer hexane layer to 250 mL separatory funnel and re-extract residue twice with 45 mL hexane, shaking vigorously 1 min as before. Add 20 mL water to pooled hexane extracts and drain off water. Shake hexane once with 100 mL 5% KOH, twice with 100 mL water, and once with 100 mL 55% ethanol. Evaporate in 500 mL boiling flask under reduced pressure at 50°C. Add 5 mL ethanol and re-evaporate to remove traces of water.

HPLC of Extracts from Milk on Silica Column

Set pen recorder at full scale deflection (FSD) of approximately 0.02 and, if possible, monitor also at lower sensitivity as in Figure 1. Integrator is not required but timer is needed to measure elution times of peaks. Degas solvent immediately before use by placing flask in sonic bath and applying 5–10 s bursts of suction from water aspirator. Degassing process may have to be repeated several times a day. Set pressure limiter

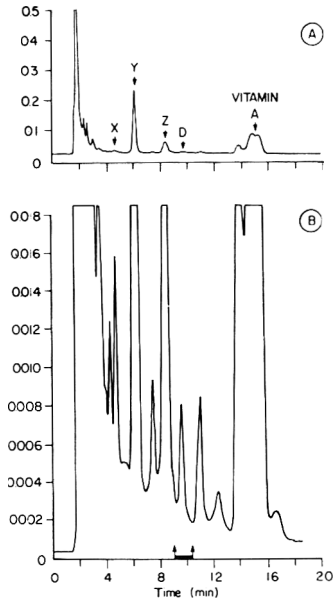


Figure 1. Typical liquid chromatogram of milk unsaponifiable lipids on 15 cm Supelcosil LC silica column, recorded simultaneously at low (A) and high (B) sensitivity. Position of elution of vitamin is indicated by D in (A), and portion of eluate collected is indicated by arrows in (B). Peaks Y and Z are characteristic of milk. Improperly washed extracts yield large peak at × (see text). Solvent system: 0.5% isopropanol in cyclohexane-hexane (1 + 1). Flow rate: 1 mL/min. Ordinate indicates absorbance at 254 nm.

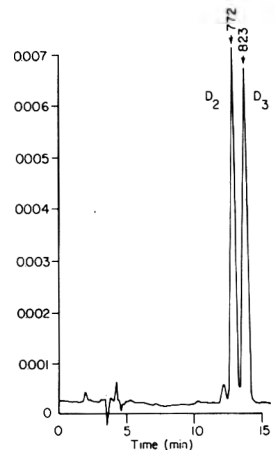


Figure 2. Liquid chromatogram of standards of vitamin D_2 and D_3 on 5 μm Radial-Pak column. Retention times marked in seconds. Solvent system: methanol. Flow rate: 1 mL/min. Ordinate indicates absorbance at 254 nm.

on pump to 1000 psi.[1] Inject 80 μL vitamin D standard solution (40 IU vitamin D/mL in solvent system) and note time of elution of vitamin (identical for vitamins D_2 and D_3; retention time of peak ca 10–12 min). Transfer sample with hexane to 15 mL centrifuge tube and evaporate under nitrogen. Transfer to 1 mL Reactivial with hexane and evaporate. Dissolve in 100 μL solvent system and inject 80 μL, taking care not to lose solvent by evaporation. Identify peaks Y and Z (Figure 1), using FSD = 0.5 AU, and then increase sensitivity FSD = 0.02 AU and collect eluate containing vitamin D in 5 mL Reactivial.

In routine analysis, increase flow rate to 3 mL/min after collection of vitamin D to accelerate elution of remaining peaks. A sample can then be applied every 15 min.

Column may be washed at weekly intervals or after analysis of 50 samples with 20 mL chloroform–methanol (2 + 1).

Reverse Phase Chromatography

Connect detector to integrator and set pen at FSD = 0.01 AU. Inject 60 μL standards (40 IU vitamin D/mL in solvent system) and measure areas under peaks. Repeat until consistent values are obtained. Vitamin D_2 should separate from vitamin D_3. Radial-Pak column is recommended (Figure 2) because of the complete separation, but satisfactory results can be obtained with other columns (Figure 3). Evaporate eluates from silica column under nitrogen, dissolve in 80 μL solvent system, and inject 60 μL. Identify form of vitamin D from retention time and measure areas under peak (Figure 3).

Saponification and Extraction of Infant Formula

Mix or dilute formula with water to give final concentration of 300–400 IU vitamin D/L. Pipet 10 mL diluted formula and 5 mL water into 50 mL flask for saponification and extraction as described for milk.

Saponification and Extraction of Margarine

Melt and mix ca 50 g margarine in beaker at 40°C. Dissolve 5.0 g in 100 mL hexane and evaporate 5 mL aliquot in 50 mL stoppered flask.

[1] Cyclohexane has been observed by others to freeze in HPLC equipment operating at high pressures, and pumps have been seriously damaged. Caution is therefore needed when experimenting with this solvent. This problem has not been encountered in the present method in which the operating pressures with the Supelcosil column are below 500 psi.

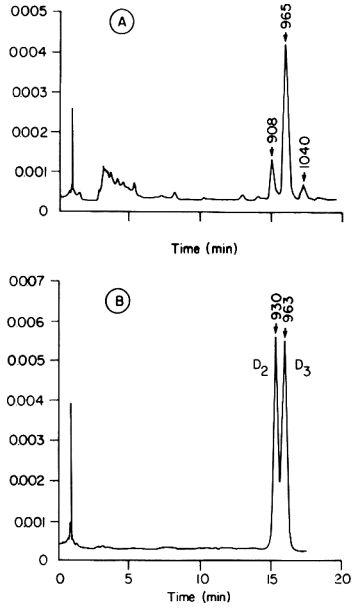


Figure 3. (A) Reverse phase liquid chromatogram of fraction from milk, collected as in Figure 1, on 25 cm Spherisorb ODS column compared with (B) standards of vitamins D_2 and D_3. Retention times marked in seconds. Milk contained 24 IU vitamin D_3/100 mL according to area under peak at 965 s. Solvent system: methanol-acetonitrile (1 + 9). Flow rate: 1 mL/min. Ordinate indicates absorbance at 254 nm.

Add 15 mL 1% ethanolic pyrogallol and 2 mL aqueous KOH (1 + 1, w/v). Shake overnight at room temperature. Add 29 mL water and 5 mL ethanol, and extract as described for milk.

Cleanup of Extracts from Infant Formula and Margarine on Alumina

Prepare 5 cm columns of alumina in Pasteur pipets and wash with 5 mL 1% isopropanol in hexane as described above (see Alumina). Dissolve unsaponifiable lipids from sample (after evaporation in 500 mL boiling flask) in 2 mL isopropanol-hexane and transfer with Pasteur pipet to alumina column, collecting eluate in 15 mL centrifuge tube. Rinse flask with another 2 mL isopropanol-hexane and transfer rinse to column. Repeat this process with 4 more portions (each 2 mL) of isopropanol-hexane and collect entire 12 mL as it passes from column.

HPLC of Extracts from Infant Formula and Margarine

Evaporate eluate from alumina column under nitrogen and follow procedures for HPLC of

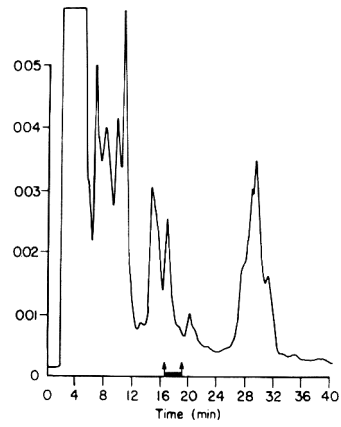


Figure 4. Liquid chromatogram on silica of unsaponifiable lipids from margarine after cleanup on column of alumina. Conditions as in Figure 1 except 0.25% isopropanol used in solvent system. Portion of eluate collected is indicated by arrows. Ordinate indicates absorbance at 254 nm.

extracts from milk, but use 0.25% isopropanol in cyclohexane-hexane (1 + 1) as solvent system on silica column. Vitamin D should be eluted after 17–19 min (Figures 4 and 5). After collection of vitamin D, increase flow rate to 3 mL/min to speed elution of later peaks. A sample can thus be applied every 40 min.

Calculation of Results

Concentration of each vitamin in milk, C, is given by:

$$C(\text{IU}/100\text{ mL}) = (PA/PA') \times C \times 0.08 \times (100/80) \times (100/15) = (PA/PA') \times C \times 0.667$$

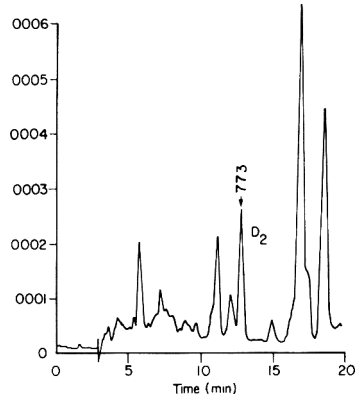


Figure 5. Reverse phase liquid chromatogram of fraction from margarine, collected as in Figure 4, on Radial-Pak column. Conditions as in Figure 2. Margarine contained 629 IU vitamin D_2/100 g. Ordinate indicates absorbance at 254 nm.

where PA = area beneath peak for sample; PA' = area beneath peak for standard; C = concentration of standard (IU/mL).

Concentration in formula and margarine is:

$C(\text{IU/g or mL}) = (PA/PA') \times C \times 0.08 \times (100/80) \times (1/W) = (PA/PA') \times (C/W) \times (1/10)$

where W = the weight or volume of the sample placed in the saponification flask.

Results and Discussion

The present methods for fortified milk, margarine, and formula were developed in stages over a period of several years from one described previously for milk, which originally involved saponification, chromatography on HAPS, and HPLC on silica (12).

In the new method, vitamin D was measured in milk in 4 steps: saponification, extraction, HPLC on a column of silica, and HPLC in a reverse phase system. A cleanup step with a small alumina column was introduced and minor changes were made in the HPLC systems for the analysis of margarine and formula.

The saponification step was retained from the previous method because it proved to be an efficient way to remove a large amount of lipid. Another advantage of saponification was that it simplified the extraction of milk and formula by solubilizing their constituents and releasing the added vitamin from any protective matrix. The alternative, extraction of lipid without saponification, was rejected not only because it yielded bulky extracts requiring unreasonably large chromatography columns for analysis, but also because with milk and formula it was at least equally laborious.

The vitamins are thermally isomerized to previtamins (17) and this interconversion must be considered in the design of the saponification step, especially when it includes refluxing in aqueous alcohol. The previtamins are well separated from the vitamins in many straight phase and reverse phase chromatography systems (18) and thus in most methods of analysis for vitamin D they are not recovered for direct measurement. Some workers have overlooked this source of error when measuring vitamin D in foods whereas others have obtained correction factors by passing standards through their procedures (6, 7) or by analyzing heated solutions of the vitamin (10). In the present method, as in our previous procedure (12), many of the problems associated with isomerization were avoided by performing the saponification at room temperature. This technique was more convenient than the usual hot saponification because it required less equipment, and the overnight delay was of no consequence in routine analysis. Ethanol was recommended in preference to other alcohols in spite of the fact that its purchase is sometimes subject to restrictions. Methanol is more readily available in many laboratories but it was discovered that soaps tended to precipitate from it during saponification. When propanol or higher alcohols were used, the saponification mixture often separated into 2 phases.

The extraction step was greatly improved in the new method by substituting hexane for ethyl ether. Ether is hazardous because of its volatility, flammability, and tendency to form peroxides. The requirement that it be freshly distilled adds to the risks and inconvenience in its use. In the development of the present method, a procedure was devised for the extraction of vitamin D from the saponification digest with relatively small volumes of hexane. Hexane is a convenient solvent which is not dangerously volatile at room temperature. It can be evaporated in a reasonable time at temperatures below 50°C under reduced pressure and thus its use does not lead to significant isomerization of vitamin D.

The proportions of ethanol, water, and hexane in the extraction steps were carefully chosen to avoid the formation of emulsions and the extraction of lipids more polar than vitamin D. Considering the solvents alone, addition of the quantities used in each step reveals that 45 mL hexane were shaken with approximately 30 mL water and 20 mL ethanol (i.e., 40% ethanol) in the first extraction. Tests with standards revealed that insignificant amounts of vitamin D_3 (cholecalciferol) were extracted from hexane by aqueous ethanol when the ratio of ethanol to water was less than 6:4 (see below).

In tests of the completeness of extraction of lipids after saponification, it was discovered that prolonged vigorous shaking was as efficient as several short extraction steps; in the present method 3 extractions of specified duration were therefore substituted for the 5 or more used previously.

After washing with water, the extract was shaken with aqueous KOH solution to remove traces of polar constituents which probably included soaps (19). Before this step was introduced, it was not possible to chromatograph even the extracts from milk on small HPLC columns without some form of preliminary purification, such as filtration through a disposable column of alumina prepared in a Pasteur pipet. Otherwise, a large tailing peak was observed during HPLC on silica at the position marked X in Fig-

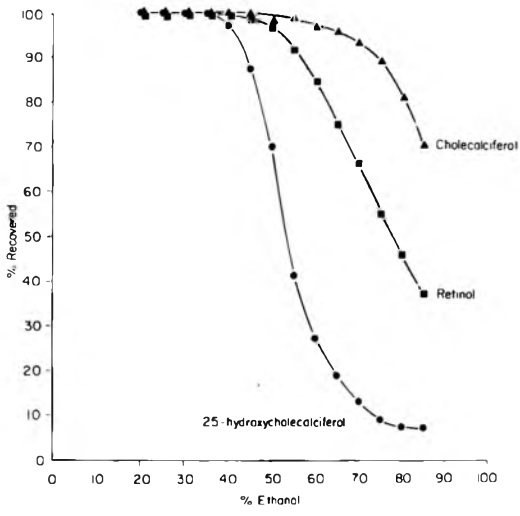


Figure 6. Partition of vitamin D_3 (cholecalciferol), retinol, and 25-hydroxycholecalciferol between equal volumes of hexane and aqueous ethanols of various strengths. Ordinate indicates percentage of each vitamin recovered in the hexane layer after vigorous shaking.

ure 1, and the performance of the column deteriorated rapidly.

The wash with 55% ethanol had a less dramatic, and in fact barely visible, effect on the subsequent HPLC but it was included to remove polar material which could be present in the samples or reagents. The design of this step was based on a study of the partition of vitamin D between hexane and aqueous alcohols. In a series of experiments, hexane solutions of vitamin D (3 mL) were shaken with equal volumes of aqueous ethanols (20–85%) and then separated by centrifugation. The recovery of vitamin D in the hexane layer was measured by injecting aliquots (50 μL) into an HPLC system (silica column). The results indicated that little vitamin D (cholecalciferol) was extracted from the hexane layer when the strength of the alcohol was below 60% (Figure 6). More polar lipids, however, were removed by 50% ethanol. The behavior of dihydroxy compounds, for example, was indicated by similar tests with 25-hydroxycholecalciferol (Figure 6).

The washed extract from milk was then evaporated rapidly at 50°C (conditions in which thermal isomerization to previtamin is negligible) and subjected to HPLC first on silica column and second in a reverse phase system, which together provided a degree of separation reminiscent of "2-dimensional" paper or thin layer chromatography. The extract was poorly soluble in methanol but more readily soluble in hexane, which is one reason why the silica column, rather than the reverse phase column, was used first. The extract was actually dissolved in the solvent system (isopropanol, hexane, cyclohexane) rather than hexane before chromatography, not merely because this is accepted practice in chromatography, but because the isopropanol and cyclohexane significantly improved the solubility of the sample which was at this stage mainly sterols. Cyclohexane was included in the solvent system only for this reason, that is, to improve the solubility of the sample; it had no other effect of any consequence on the chromatography other than that it increased the back-pressure because of its relatively high viscosity and it caused a small change in retention times because of its polarity.[1]

Vitamin D was separated from the bulk of the unsaponifiable lipids from milk on the silica column and was detectable, but not measurable, in a recording of the absorption at 254 nm. Thus a peak or shoulder was observed at the retention time of vitamin D in a "valley" between large peaks eluted at the beginning of the chromatography, and those of vitamin A and its isomers eluted later (Figure 1). It can be calculated that there is sufficient vitamin D in properly fortified milk to account for a substantial part of the material observed at this retention time, but it is not possible to obtain accurate measurements of the level of the vitamin without further chromatography. This is emphasized because often a sharp peak was observed during chromatography and there was a strong temptation to assume that it truly represented the vitamin D content; actually the appearance proved to be deceptive, and significant and variable amounts of other absorbing substances were always present. The chromatography was performed on other modern efficient columns in addition to Supelcosil, but small changes in the concentration of isopropanol in the solvent system were necessary. Columns which have been used successfully include LiChrosorb Si 60, 5 μm, and Waters Radial-Pak B.

It was important in this step to collect the vitamin D fraction at precisely the correct time but this was a straightforward procedure when the pump was functioning properly. However, when the flow rate was erratic, because of leaks or malfunctioning pistons, collection of the vitamin D fraction was difficult. It was therefore important to keep the equipment in good condition and to degas the solvents to avoid cavita-

Table 1. Determination of vitamins D_2 and D_3 (IU/100 mL or IU/100 g) in infant formulas

Sample[a]	Label claim	Result	Percent label claim	Form of vitamin
1M	328	461	140	D_2
2M	288	403	140	D_2
3M	86	121	141	D_2
4M	43	64	149	D_2
5S	84	94	112	D_2
6M	304	330	108	D_3
7M	304	337	111	D_3
8M	40	41	102	D_3
9M	80	86	108	D_3
10S	80	67	84	D_3
11M	40	40	100	D_3
12S	40	32	80	D_3
13M	80	76	95	D_3
14M	330	449	136	D_3

[a] M indicates milk base, S indicates soy base. Each sample analyzed once.

tion in the pumps. Usually milk samples gave similar elution patterns and, with a little experience, the position of vitamin D was recognizable even when, for one reason or another, the duration of the chromatography changed. The substances responsible for peaks Y and Z have not been identified but they were characteristic of milk, and their retention times and those of smaller peaks could be used to monitor the reproducibility of the chromatography.

The eluted vitamin D was evaporated under nitrogen, again taking care to avoid unnecessary heating, and then a portion was rechromatographed on a reverse phase column. This system separated the vitamin D from remaining traces of absorbing substances and also separated vitamin D_2 from vitamin D_3. Again, several modern columns have been used successfully for this step. The most efficient separation of the 2 vitamins was achieved with a 5 μm Waters Radial-Pak A column eluted with methanol, a system recommended to us by the Applications Laboratory of Waters Scientific Ltd.

The analysis of fortified milk was straightforward. Well separated peaks were obtained in the reverse phase system and the identification and measurement of the vitamin presented few problems. Reproducibility was good and in a test, a coefficient of variation of 3% was obtained in the analysis of 8 replicate samples. Recovery of vitamin D added to samples of unfortified milk just before saponification was 96–99%.

The analysis of infant formula and margarine was more demanding than that of milk and a cleanup column of alumina was used before HPLC. Improved separation of vitamin D from interfering substances was also achieved on the HPLC silica column by weakening the polarity of the solvent system at the cost of increased time of analysis. Because formula and margarines can contain a wide variety of ingredients, the HPLC recordings varied from one sample to another. Sometimes many peaks were observed in the final reverse phase chromatography and the identification of vitamins D_2 and D_3 was less certain than in the analysis of milk. These problems were attributable in large part to substances chromographing with retention times similar to those of vitamins D_2 and D_3 on the silica column, which were encountered in corn oil, soybean oil, and other vegetable oils.

Small peaks running close to vitamin D also had an adverse effect on quantitative measurements, especially those based on areas. Results were therefore usually calculated from peak heights as well as from areas and when a large discrepancy (i.e., ratio >1.3) was observed between the 2 values, the analysis was considered to be invalid and it was repeated.

Although the results were routinely calculated from areas, in the analysis of margarine, peak heights sometimes appeared to be more consistent. The coefficients of variation for the analysis of formula and margarine were 6% (5 replicates) and 9% (6 replicates), respectively, based on areas and 9 and 5%, respectively, based on peak heights.

The results of the analysis of various samples of formula and margarine are listed in Tables 1 and 2. Margarines contained only vitamin D_2. Vitamin D_2 and vitamin D_3 were encountered in formula.

As discussed previously (12), the currently accepted definition of the International Unit (IU)

Table 2. Determination of vitamin D_2 (IU/100 g) in margarines

Sample[a]	Type[b]	Label claim	Result	Percent label claim
1S	S	660	680	103
2S	S	660	807	122
3H	S	550	714	130
4S	C	660	659	100
5H	C	660	618	94
6H	C	550	479	87
7S	F	550	606	110
8S	V	660	881	133
9S	V	660	625	95
10H	V	550	332	69
11H	V	550	629	114
12H	V + M + A	550	415	75

[a] S indicates soft margarine, H indicates hard margarine. Each sample analyzed once.
[b] Coded: S—soybean; C—corn; F—sunflower; V—mixed vegetables; M—marine; A—animal.

as 0.025 μg vitamin D_2 or 0.025 μg vitamin D_3 does not take into account the difference between the vitamins in molecular weight and it complicates the calculation of the results of analyses. A more logical definition, first suggested by Norman (20), is 65 pmoles (i.e., 0.0250 μg vitamin D_3 or 0.0258 μg vitamin D_2). If it is also accepted that the molecular extinction coefficients of both vitamins are 18 300 in ethanol (21), it can be calculated that the absorbance of a solution containing 100 IU/mL is 0.119 (i.e., $100 \times 65 \times 10^{-12} \times 18\,300 \times 10^3$). This relationship is useful in HPLC analysis because it simplifies the spectrophotometric assay of standard solutions. Moreover, as the areas under peaks are proportional to absorbance, it follows that similar factors can be used for both vitamin D_2 and vitamin D_3 when the results are calculated in IU.

Acknowledgment

We thank Cécile Robitaille, Health Protection Branch Laboratories, Longueuil, Québec, for valuable advice and assistance in the development and evaluation of our procedures.

References

(1) Bell, J. G., & Christie, A. A. (1974) *Analyst* **99**, 385–396
(2) Panalaks, T. (1971) *J. Assoc. Off. Anal. Chem.* **54**, 1299–1303
(3) Ray, A. C., Dwyer, J. N., & Reagor, J. C. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 1296–1300
(4) Ruckemann, H., & Ranfft, K. (1977) *Z. Lebensm. Unters. Forsch.* **164**, 272–273
(5) Lein, D. G., Campbell, H. M., & Cohen, H. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1149–1152
(6) Ali, S. L. (1978) *Fresenius Z. Anal. Chem.* **293**, 131–134
(7) Egaas, E., & Lambertsen, G. (1979) *Int. J. Vitam. Nutr. Res.* **49**, 35–42
(8) Mulder, F. J., De Vries, E. J., & Borsje, B. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 1031–1040
(9) Antalick, J. P., Debruyne, H., & Faugere, J. G. (1977) *Ann. Falsif. Expert. Chim.* **70**, 497–502
(10) Knapstein, H., Puchel, P., & Scholz, H. (1979) *Fette Seifen Anstrichm.* **81**, 121–126
(11) Cohen, H., & Lapointe, M. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1158–1162
(12) Thompson, J. N., Maxwell, W. B., & L'Abbé, M. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 998–1002
(13) Henderson, S. K., & Wickroski, A. F. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 1130–1134
(14) Henderson, S. K., & McLean, L. A. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 1358–1360
(15) Adachi, A., & Kobayashi, T. (1979) *J. Nutr. Sci. Vitaminol.* **25**, 67–78
(16) Cohen, H., & Wakeford, B. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1163–1167
(17) Keverling Buisman, J. A., Hanewald, K. H., Mulder, F. J., Roborgh, J. R., & Keuning, K. J. (1968) *J. Pharm. Sci.* **57**, 1326–1329
(18) De Vries, E. J., Zeeman, J., Esser, R. J. E., Borsje, B., & Mulder, F. J. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 129–135
(19) Benham, G. H. (1944) *Can. J. Res.* **22B**, 21
(20) Norman, A. W. (1972) *J. Nutr.* **102**, 1243–1246
(21) Shaw, W. H. C., Jefferies, J. P., & Holt, T. E. (1957) *Analyst (London)* **82**, 2–7.

DRUG RESIDUES IN ANIMAL TISSUES

Screening Test for Sulfamethazine and Sulfathiazole in Swine Liver

OWEN W. PARKS
U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118

A screening method is described for estimation of sulfamethazine and sulfathiazole residues in swine livers. Tissues are homogenized with $CHCl_3$-ethyl acetate (1 + 1). The drugs are extracted from the organic solvent with pH 10 carbonate buffer and back-extracted into dichloromethane, as an ion-pair with tetrabutylammonium hydroxide, without pH adjustment. Following evaporation of the solvent, the residue is dissolved in methanol, subjected to thin layer chromatography, and detected by the Bratton-Marshall reaction. Recoveries of sulfamethazine and sulfathiazole, determined by high pressure liquid chromatography, were 50.8 and 42%, respectively, with coefficients of variation of 4.2 and 4.7%.

Sulfa drugs are widely used in animal production. In swine, sulfamethazine and, to a lesser degree, sulfathiazole, are the drugs of choice. Although federal regulations require animal withdrawal from drug use long enough to limit residues in swine livers to 0.1 ppm, the Food Safety and Inspection Service (FSIS), U.S. Department of Agriculture, reported (1) that 4.2% of the livers examined in 1980 were in violation of the tolerance level. The violation rate, although unacceptable, represents a significant decrease compared with the 15% rate determined in 1978 (2).

FSIS and the Food and Drug Administration have relied primarily on a modification of the Tishler et al. procedure (3) to detect and quantitate sulfa drug residues in swine livers. The limitations of this procedure with regard to time for analyses (5–7 samples/analyst/day) and reliability of results are well established (4). The economic advantage of a more rapid and reliable screening procedure, especially in view of the decrease in violations in the past 2 years, is evident.

To be totally effective, a laboratory screening procedure must be relatively rapid, capable of detecting drug residues at the violative level, free of false positive results, and must tentatively identify the contaminating drug and demonstrate reproducible results over a wide range of concentrations. The screening methods currently proposed for detecting sulfa drugs in swine tissues fail to meet one or more of these criteria. The procedure for detecting sulfamethazine and sulfathiazole in swine livers presented here was designed specifically to meet these requirements.

Received September 9, 1981. Accepted November 6, 1981.

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.

METHOD

Reagents

(a) *Solvents.*—Ethyl acetate and dichloromethane (DCM) (Distilled-in-Glass®, Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442); $CHCl_3$, "Baker Analyzed" reagent (J. T. Baker Chemical Co., Phillipsburg, NJ 08665).

(b) *Tetrabutylammonium hydroxide (TBAH).*—Aldrich Chemical Co., Inc., Milwaukee, WI 53233. 40% aqueous solution.

(c) *N-1-(Naphthyl)ethylenediamine (NEDA) dihydrochloride.*—Sigma Chemical Co., St. Louis, MO 63178. 0.4% methanol solution.

(d) *0.1M Carbonate buffer.*—pH 10. Prepare from 0.1M solutions of sodium carbonate and sodium bicarbonate.

(e) *Sulfamethazine.*—American Cyanamid Co., Princeton, NJ 08540.

(f) *Sulfathiazole.*—Sigma Chemical Co.

Apparatus

(a) *Tissue grinder.*—Brinkmann Polytron® homogenizer (Brinkmann Instruments Inc., Westbury, NY 11590).

(b) *Centrifuges.*—International clinical centrifuge-rotor No. 273 (International Equipment Co., Needham Heights, MA 02194); Sorvall superspeed centrifuge-type SS-1 rotor (Ivan Sorvall, Inc., Norwalk, CT).

(c) *High pressure liquid chromatographic (HPLC) system.*—Laboratory Data Control (Riviera Beach, FL 33404) Constametric pump controlled by gradient master programmer and connected to

0004-5756/82/6503-0632-03$01.00

Schoeffel Model SF770 Spectroflow monitor operated at 254 nm. Rheodyne 7120 sampling valve. Column, 30 cm × 4 mm id μBondapak C_{18}. Mobile phase, water–methanol–acetic acid (80 + 20 + 0.5) with 0.1% TBAH. Samples eluted isocratically at flow rate of 1 mL/min.

(d) *Vortex stirrer.*—Super mixer (Lab-Line Instruments, Inc., Melrose Park, IL 60160).

(e) *Thin layer chromatographic (TLC) system.*—2.5 × 10 cm glass plate with 250 μm layer of silica gel G (Analtech, Newark, DE 19711). Developing solvent, ethyl acetate–methanol (4 + 1).

Procedure

Weigh 2.5 g ground frozen liver (5) into 50 mL polypropylene centrifuge tube. Let liver partially thaw. Add 16 mL cooled (4.4–10°C) $CHCl_3$–ethyl acetate (1 + 1) and blend 30 s with Polytron homogenizer at low speed. Centrifuge 2 min at 3000 rpm. Remove solvent with disposable Pasteur pipet and filter through small plug of glass wool packed in disposable super Pasteur pipet, collecting filtrate (ca 12.5 mL) in 15 mL screw-cap centrifuge tube. Add 2 mL pH 10 buffer and shake carefully in rocking motion for 2 min. Centrifuge 2 min at 2500 rpm. Carefully transfer aqueous phase to a second 15 mL screw-cap centrifuge tube with aid of Pasteur pipet. Add 30 μL TBAH solution and vortex-mix 15 s. Add 13 mL DCM and shake vigorously 3 min. Centrifuge 2 min at 2500 rpm. Carefully pour contents of centrifuge tube into 60 mL separatory funnel containing 2 mL DCM in manner to prevent mixing of phases. Let stand 1 min. Slowly draw off DCM phase into 23 mL screw-cap specimen vial. Evaporate solvent in 9 mL screw-cap specimen vial at 50°C under stream of nitrogen, transferring entire solution with disposable Pasteur pipet.

Dissolve residue in 0.2 mL methanol and spot 10 μL in 1.0–1.5 μL increments, drying spot with aid of stream of nitrogen between applications. Maintain diameter of spot at ≤4 mm. Develop plates 0.5 cm from origin. Dry plates in 60°C forced air oven and redevelop plates to height of 3 cm from origin. Dry plates in forced air oven before spraying with visualizing reagents.

Modified Bratton-Marshall (BM) Color Development (6)

Expose plates for 5 s to nitrous acid vapors generated by addition of sodium nitrite to 8% aqueous phosphoric acid (use hood). Allow 10 s for excess nitrous acid to dissipate. Spray with NEDA reagent to produce pink spots.

Recovery Studies

Absolute drug recoveries by the screening procedure were determined by HPLC of liver extracts. Drug-free liver samples were spiked by injection into tissue with sulfamethazine and sulfathiazole at 0.10, 0.20, 0.44, and 0.60 ppm before analysis. In addition, extracts of drug-free livers were spiked with the various concentrations of the drugs to prepare standard curves. Drug-free liver extracts served as a control for both. Residues of liver extracts were dissolved in 0.08 mL methanol, and 0.32 mL water–acetic acid–TBAH (80 + 0.5 + 0.1) was added. Mixture was vortexed 30 s, followed by centrifugation for 1 min at 2500 rpm. Thirty microliter samples were injected onto the HPLC column. Recoveries were determined on the basis of peak heights. Retention times were 10.5 min for sulfamethazine, and 7.0 min for sulfathiazole.

Results and Discussion

The method described has been successfully applied to swine livers spiked with sulfamethazine and sulfathiazole, as well as livers containing naturally incurred sulfamethazine at or above the violative level. The use of ethyl acetate–methanol (4 + 1) as the TLC developing solvent, together with restricting the diameter of sample spot applied at the origin to 4 mm and solvent migration to 3 cm, results in compact bands (sulfamethazine, R_f 0.9; sulfathiazole, R_f 0.8) on the TLC plates. As a result, this enables the detection of 0.02 ppm of the drugs in liver.

The use of the pH 10 carbonate buffer for extracting the sulfa drugs from an organic solvent, rather than the use of an acid solution as in most proposed methods, serves 2 important purposes. First, it limits potentially interfering BM-positive aromatic amines (e.g., procaine) to amphoteric compounds. Second, and more important, by the technique of ion-pairing with TBAH (7), the sulfa drugs are back-extracted into an organic solvent without the need for pH adjustment of the aqueous solution.

Analyses of approximately 25 livers consistently resulted in the detection of an unknown BM-positive compound in varying concentrations. The unknown (R_f 0.6), characterized by its slow color development relative to sulfamethazine and sulfathiazole, does not, however, interfere with determining the presence or absence of the drugs. The identification of the unknown is currently under investigation.

The absolute percent recoveries of sulfa-

Table 1. Recovery of sulfathiazole and sulfamethazine added to swine liver

	Recovery,[a] %	
Added, ppm	Sulfathiazole	Sulfamethazine
0.1	44.0	50.5
0.1	45.8	50.8
0.1	40.5	50.1
0.1	40.9	46.5
0.2	41.0	47.7
0.2	43.2	49.7
0.2	39.6	53.8
0.2	44.4	55.1
0.44	40.7	52.1
0.44	38.5	50.1
0.44	41.3	51.2
0.44	42.9	48.9
0.6	42.6	54.1
0.6	39.6	51.0
0.6	43.7	50.8
0.6	43.1	50.8

[a] HPLC recovery, based on actual quantity of drug recovered relative to quantity of drug added.

methazine and sulfathiazole, as determined by HPLC, from 16 swine liver samples spiked with concentrations ranging from 0.1 to 0.6 ppm are presented in Table 1. The mean recoveries were 50.8 and 42.0%, respectively, with coefficients of variation of 4.2 and 4.7%. Because relatively consistent recoveries were obtained over a range of sulfa drug concentrations, an internal standard is not considered necessary in the procedure. Furthermore, the approximate concentration of the contaminating drug can be determined by comparing the color intensities of positive samples with that of appropriate drug standards. Analysts, therefore, can determine those positive samples which merit more lengthy and elaborate quantitation and confirmation studies.

In the method outlined, higher absolute recoveries of the sulfa drugs were sacrificed to minimize the time of analysis. In this respect, an individual analyst who performs 4 concurrent analyses can complete 20 samples in an 8-h period.

Acknowledgment

The author thanks Raymond B. Ashworth, FSIS, Methods Development Laboratory, Beltsville, MD, for providing liver samples containing naturally incurred levels of sulfamethazine, and Walter Fiddler for helpful suggestions during the development of this method.

References

(1) *Food Chemical News* (1980) **22**(41), 4
(2) Goyan, J. E. (1980) *Fed. Regist.* **45**(189), 63930
(3) Tishler, F., Sutter, J. L., Bathish, J. N., & Hagman, H. E. (1968) *J. Agric. Food Chem.* **16**, 50–53
(4) Horwitz, W. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 104–130
(5) Goodspeed, D. P., Simpson, R. M., Ashworth, R. B., Shafer, J. W., & Cooke, H. R. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 1050–1053
(6) Bratton, A. C., & Marshall, E. K. (1939) *J. Biol. Chem.* **128**, 537
(7) Gyllenhaal, O., Tjarnlund, U., Ehrsson, H., & Hartvig, P. (1978) *J. Chromatogr.* **156**, 275–283

Radioimmunoassay of Oxfendazole in Sheep Fat

CLINTON NERENBERG, IRENE TSINA, and SHAIKH MATIN
Syntex Research, Institute of Pharmacology and Metabolism, Department of Analytical and Metabolic Chemistry, Palo Alto, CA 94304

Oxfendazole (methyl 5-(phenylsulfinyl)-2-benzimidazole-carbamate) is a broad spectrum anthelmintic agent designed for use in food-producing animals. A simple radioimmunoassay (RIA) for determination of oxfendazole in plasma was modified for determining oxfendazole in sheep fat. Fat tissue was enzymatically hydrolyzed to an oily residue with collagenase-hyaluronidase, and oxfendazole was then extracted into an acidified aqueous phase. An aliquot of this phase was used directly for RIA. Bound radioactivity was separated from free by using polyethylene glycol-bovine gamma globulin because oils and other components in the aqueous aliquot preclude the use of charcoal for the separation. The lower limit of sensitivity of the assay is 0.003 ppm. Accuracy experiments carried out in the range 0.01–0.5 ppm gave a regression line of y (ng/g) = 0.91x (ng/g) + 2.89, with r = 0.99. Fat tissue derived from sheep given an oral dose of 6.0 mg/kg was analyzed by this method and by a high pressure liquid chromatographic (HPLC) method. Values obtained by the 2 methods agreed well.

Oxfendazole (methyl 5-(phenylsulfinyl)-2-benzimidazole-carbamate), a new anthelmintic agent, is active against a broad spectrum of gastrointestinal nematodes, lungworms, and tapeworms found in cattle, sheep, and pigs (1). To be able to administer this drug to animals that provide edible tissues for man, it is important to determine tissue residue levels with respect to time so that a withholding period can be established before human consumption of the tissue can safely begin. One of the edible tissues is fat and, to determine oxfendazole in this tissue, an analytical method was required with sufficient sensitivity and utility to permit the analysis of a large number of samples in a relatively simple manner. We modified a simple radioimmunoassay (RIA) for determining oxfendazole in plasma (2) and used it to determine residues in fat tissue. This assay was validated by a high pressure liquid chromatographic (HPLC) method (3). The RIA method has sufficient sensitivity to provide data on residue depletion of oxfendazole in fat tissue.

Received July 22, 1981. Accepted November 20, 1981.

Experimental

Materials and Apparatus

Collagenase (CLS, Type I, Worthington Biochemical Corp.), hyaluronidase (Type II, ovine testes, Sigma Chemical), bovine gamma globulin (Bovine, Fraction II, Calbiochem), and polyethylene glycol (Carbowax 6000) were purchased commercially. The radioimmunoassay buffer was 0.05M phosphate-buffered saline, pH 8.0, with or without 0.1% gelatin. The tissue-processing buffer was 0.1M phosphate-buffered saline, pH 7.0. Labeled oxfendazole was synthesized in our laboratory and was tritiated at the 4′-phenyl position with a specific activity of 24–27 Ci/mmole.

The centrifuge (International Equipment Co., Model HN-S) was a bench top model with swing-out head. Liquid scintillation counting was done in a scintillation spectrometer (Packard Tri-Carb, Model 3330), using commercially available scintillation fluid (Aquasol or Oxi-fluor-H_2O, New England Nuclear).

Antisera Production

Preparation of the conjugate and immunization of the animals has been described in a previous publication (2).

Tissue Preparation

Approximately 1.0 g frozen fat tissue was weighed to the nearest mg. The tissue was finely minced with a razor blade on a glass plate and then transferred to a scintillation vial. To the vial was added 5.0 mL collagenase–hyaluronidase enzyme mixture. This enzyme mixture was prepared by dissolving the enzymes in 0.1M phosphate-buffered saline, pH 7.0, such that 5 mg collagenase and 3 mg hyaluronidase are contained in 5.0 mL. The vials were loosely capped and the fat tissue was then incubated 2 h at 37°C. After completion of the incubation, 2 drops of concentrated HCl were added to each vial and the vials were heated 30 min in a boiling water bath. The contents of the hot vials were transferred to 13 mL centrifuge tubes, the vials were washed with an additional 1.0 mL 0.1N HCl, and the washings were added to the cen-

0004-5756/82/6503-0635-05$01.00

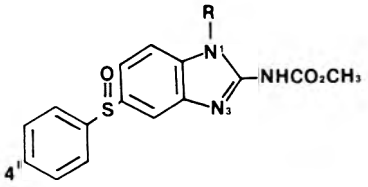


Figure 1. Structure of oxfendazole. R = H.

trifuge tubes. The tubes were kept in a hot water bath and, while the upper aqueous layer was still liquid, the centrifuge tubes were vigorously vortexed 15–20 s. The tubes were then centrifuged, making sure that the oily layer on top had not solidified. If it had solidified, the tubes were warmed in a hot water bath until the oily layer liquified, and then centrifuged. After centrifugation, a 1.0 mL aliquot of the aqueous layer was withdrawn and the pH was adjusted to pH 7.0–7.5 using pH paper. Usually 1 drop of 6N NaOH brings the pH within the required range. If the samples required dilution, 0.1M phosphate-buffered saline at pH 7.0 was used. At this point, a 0.1 mL aliquot was taken for RIA. Control tissue from untreated animals was processed in a manner identical to the unknown samples. A 0.1 mL aliquot was added to all tubes in the standard curve and the nonspecific binding tubes to create a medium environment chemically equivalent to the unknown samples.

Radioimmunoassay

Oxfendazole standards for the standard curve were obtained by diluting a stock solution of 1.0 mg oxfendazole in 10.0 mL propylene glycol–0.2N HCl (1 + 1). The series of standard solutions, 20, 50, 100, 200, 500, 1000, and 1500 pg/0.1 mL, were prepared in 0.05M phosphate-buffered saline, pH 8.0, without gelatin in 10.0 mL volumes and stored in the refrigerator. Gelatin was withheld from the buffer in the standards to give them a longer shelf life.

The bovine gamma globulin was prepared fresh each day by dissolving it in water at a concentration of 1.0% (w/v). Polyethylene glycol was prepared in volumes sufficient for several assays by dissolving it in water at a concentration of 30% (w/v).

A stepwise procedure for setting up the assay is as follows: Add buffer, label, standards, unknowns, antiserums, etc., to the appropriate 12 × 75 mm disposable culture tubes. Vortex all tubes 1–3 s to wash down sides and then cover with sheets of waxed film (Parafilm). Incubate overnight at room temperature. After overnight incubation, place tubes in an ice bath and add 1.0 mL bovine gamma globulin and 1.0 mL polyethylene glycol to each tube. Vortex 20 s, let stand 15 min, and centrifuge 8 min at 2000 rpm. Add 0.8 mL aliquot to the scintillation fluid. Addition of scintillation fluid causes heavy precipitate to settle before counting can begin. This can be as long as 12 h. Count for 10 min after settling is complete.

Calculations

Data can be processed manually by converting free to bound cpm. A standard curve can then be constructed and the unknowns can be determined by interpolation. We used the Rodbard and LeWald Model I computer program (5) in conjunction with a paper tape reader. This required a preprocessor program to read the paper tape, convert free to bound radioactivity, and organize the data for submission to the Rodbard program.

Accuracy and precision of the method were determined by adding known amounts of oxfendazole to sheep fat and then assaying replicates. A solution containing accurately known amounts of oxfendazole in 0.1N HCl was added to weighed amounts of tissue obtained from untreated animals. The tissue was then processed through the described procedure and the oxfendazole was measured by RIA. The measured values were used to calculate the ratios of measured to added (Table 1).

Results and Discussion

This paper describes an enzymatic digestion procedure as a tissue-processing technique, which is a departure from the more usual approach of solvent extraction. Solvent extractions usually extract oils and residues which are sometimes difficult to resolubilize into an aqueous aliquot required for RIA. These solvent extraction residues may even require further chromatographic purification to make them amenable for RIA. When several steps are involved in the procedure, corrections for procedural losses are necessary.

The technique described here was developed so that the sample aliquot could be used directly for RIA and a correction for losses was not required.

Treatment with collagenase–hyaluronidase disperses the tissue to the consistency of an oily liquid which floats to the top in aqueous buffer. Oxfendazole is fairly soluble in strong acid and this property was used to advantage in extracting it from the oily layer into the aqueous phase. By

Table 1. Accuracy and precision measurements carried out over the concentration range 0.01–0.5 ppm

Concn, ppm	Exp. No.	Oxfendazole added, ng/g	Oxfendazole found,[a] ng/g	Ratio measured: added	Group mean, measured: added	SD	CV, %
0.5	1	463.0	477.0	1.03	0.91	0.07	7.7
		470.0	391.0	0.83			
		498.0	442.0	0.89			
	2	504.0	453.0	0.90			
		487.0	430.0	0.88			
		482.0	436.0	0.90			
0.3	1	279.0	298.0	1.07	0.97	0.10	10.3
		286.0	272.0	0.95			
		288.0	240.0	0.84			
	2	278.0	251.0	0.90			
		286.0	272.0	0.95			
		281.0	310.0	1.10			
0.2	3	202.0	179.0	0.88	0.90	0.05	5.56
		208.0	179.0	0.86			
		205.0	202.0	0.98			
	4	189.0	168.0	0.89			
		190.0	177.0	0.93			
		182.0	154.0	0.84			
0.1	1	99.9	109.0	1.09	0.93	0.12	12.9
		95.8	87.0	0.91			
		94.7	77.0	0.81			
	3	93.7	91.0	0.97			
		98.7	74.0	0.75			
		97.3	102.0	1.05			
	4	98.0	99.0	1.01			
		99.6	78.0	0.78			
		90.4	90.0	1.00			
0.05	1	49.0	42.0	0.86	0.93	0.14	15.1
		48.3	38.0	0.79			
	3	46.8	44.0	0.94			
		49.8	55.0	1.10			
		45.5	54.0	1.19			
	4	48.0	40.0	0.83			
		48.9	44.0	0.90			
		48.7	41.0	0.84			
0.01	3	9.6	9.0	0.94	0.93	0.04	4.30
		9.4	8.0	0.85			
		9.5	8.7	0.92			
	4	9.7	9.0	0.93			
		9.7	9.5	0.98			
		9.5	8.7	0.92			

[a] Each value is the mean of 6 RIA replicates.

acidification with concentrated HCl to pH 1.0, about 97% (n = 6) of the oxfendazole preferentially partitioned into the aqueous phase. When the same extraction was carried out in 0.1M phosphate buffer, pH 8.0, only 29% (n = 9) partitioned into the aqueous phase. During the tissue-processing phase of method development, experiments with radioactive oxfendazole were carried out to determine whether any oxfendazole degradation had occurred as a result of the acidification or enzymatic treatment. Chromatography on thin layer chromatographic plates indicated that no radioactive components were present other than oxfendazole.

When the sample aliquot for radioimmunoassay is prepared according to the procedure described, the resulting sample contains oils and other residues which preclude the use of charcoal for separation of bound from free radioactivity. This separation was therefore carried out by using polyethylene glycol-gamma globulin as described by Twomey and Sweat (6). They used ^{125}I as the label and the precipitated pellet containing the bound radioactivity was counted directly. Because we used a tritium label in our method, the procedure was modified to accommodate the use of a scintillation spectrometer and scintillation fluid. When the scintillation fluid is added to the sample aliquot a rather heavy precipitate is formed. If this precipitate

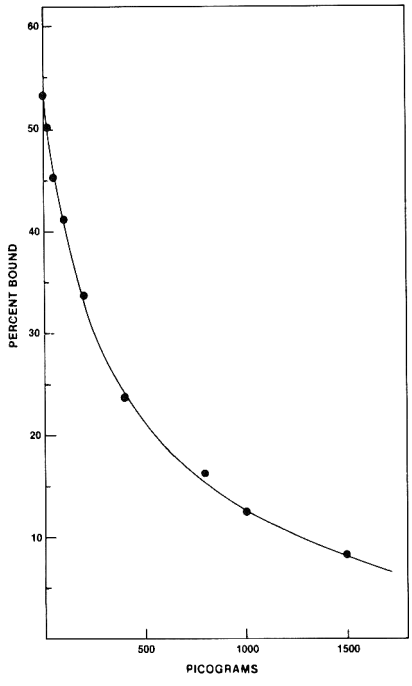


Figure 2. Plot of the displacement curve (or standard curve). The antiserum dilution was 1:1000. The reaction was carried out overnight at room temperature. Separation of bound from free was carried out with polyethylene glycol-bovine gamma globulin.

is allowed to settle, the radioactive counting is not affected. A typical RIA standard curve prepared by this procedure is shown in Figure 2.

The extent of cross-reactivities by metabolites and by compounds similar in structure to oxfendazole have been tested using the present antisera. Oxfendazole free amine, oxfendazole thioether, oxfendazole free amine thioether, and oxfendazole sulfone cross-reacted 11.3, 2.0, 0.33, and 0.25%, respectively (2).

The accuracy and precision of the method are summarized in Table 1. A regression line of y (ng/g) = $0.91x$ (ng/g) + 2.89 with a correlation coefficient of $r = 0.99$ was obtained for the concentration range 0.01–0.5 ppm. The accuracy of the method was empirically estimated to be ±15% from many experiments over a period of time.

The method was used to measure oxfendazole residue level in fat tissue of experimental animals. A total of 20 animals were given an oral suspension of oxfendazole at a dose level of 6.0 mg/kg. The animals were sacrificed in groups of four at days 3, 7, and 14. The fat tissue was analyzed by RIA and the results are listed in Table 2.

The assay was further validated by comparing the results obtained by RIA with those obtained by an HPLC method (3). In this method ox-

Table 2. RIA of oxfendazole residues in sheep fat [a]

Day	Animal No.	Oxfendazole, ppm
3	15	0.0278
	16	0.0284
	26	0.0194
	33	0.0341
Mean		0.0274
7	21	0.0053
	24	0.0067
	31	0.0057
	32	0.0059
Mean		0.0059
14	19	BDL [b]
	22	0.00360
	23	BDL
	28	BDL

[a] Animals were given a single oral dose of 6.0 mg/kg and were sacrificed in groups of four on days 3, 7, and 14.
[b] BDL = below the detectable limit (i.e., 0.003 ppm).

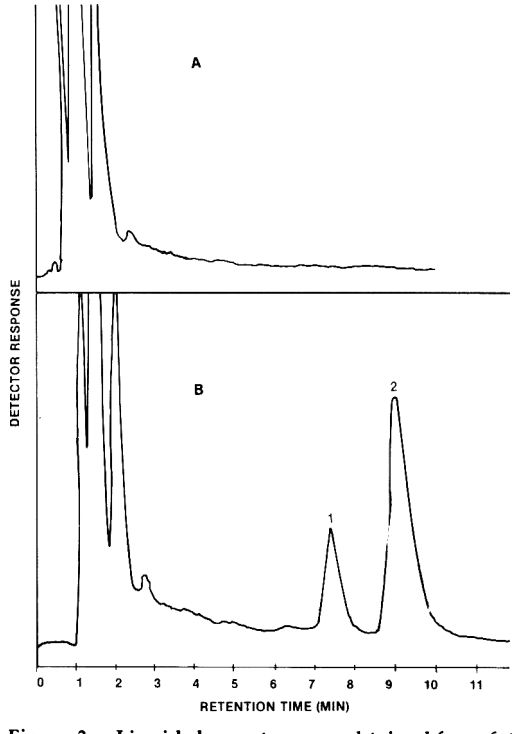


Figure 3. Liquid chromatograms obtained from fat tissue in A, untreated sheep; B, untreated tissue spiked with 0.05 ppm oxfendazole (1) and 0.1 ppm internal standard (2).

Table 3. Comparison of HPLC and RIA determinations of oxfendazole residues in sheep fat

		HPLC		RIA	
Day[a]	Animal	Found, ppm	CV, %	Found, ppm	CV, %
3	15	0.025	15.3	0.0278	10.1
	16	0.036	5.03	0.0284	15.8
	26	BDL[b]	—	0.0194	12.9
	33	0.036	5.03	0.0341	12.0
7	21	BDL	—	0.0053	26.4
	24	BDL	—	0.0067	5.9
	31	BDL	—	0.0057	26.3
	32	BDL	—	0.0059	32.2
14	19	BDL	—	BDL	—
	22	BDL	—	0.0036	—
	23	BDL	—	BDL	—
	28	BDL	—	BDL	—

[a] Day of sacrifice after dosing.
[b] BDL = below the detectable limit (i.e., 0.003 ppm for RIA and 0.025 ppm for HPLC).

fendazole was extracted from fat tissue with acetone, and the acetone extract was then defatted at low temperature. Further purification was carried out by solvent-solvent and pH partitioning. The final residue was reconstituted in methanol and an aliquot was injected onto a Waters C_{18} μBondapak reverse phase column. The mobile phase was acetonitrile-water (25 + 75). Oxfendazole was monitored by UV detection at 254 nm. The retention time was 8 min, and the lower limit of measurement was 0.025 ppm. A typical HPLC chromatogram is shown in Figure 3.

A comparison of measurements by the 2 methods, RIA and HPLC, are given in Table 3. The analytical results were in good agreement. Due to the differences in the sensitivities of the 2 methods, day 7 and day 14 samples could not be assayed by the HPLC method, whereas the RIA procedure was able to provide measureable values.

In summary, a relatively simple RIA procedure was developed for measurement of oxfendazole residues in sheep fat. The method has a lower limit of sensitivity of 0.003 ppm and an estimated accuracy of ±15%. The 0.003 ppm lower limit of sensitivity was established because the lowest point on the standard curve which can be reliably measured is 50 pg in a 0.2 mL sample aliquot.

Acknowledgments

The authors thank Colin Beard for synthesis of the oxfendazole valerate derivative, Howard Parnes for synthesis of the tritiated oxfendazole, Pamela Burton for synthesis of the oxfendazole-polylysine conjugate, and William Leitch for production of the antiserum. Recognition is also given to William Morgenstern for development of the computer programming associated with data processing. We also thank Michelle Duronslet and Linda Takenouchi for their assistance in preparing this manuscript.

References

(1) Downey, N. E. (1976) *Vet. Rec.* **90,** 267
(2) Nerenberg, C., Runkel, R. A., & Matin, S. B. (1978) *J. Pharm. Sci.* **67,** 1553
(3) Tsina, I. W., & Matin, S. B. (1981) *J. Pharm. Sci.* **70,** 858
(4) Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. (1951) *J. Biol. Chem.* **193,** 265
(5) Rodbard, D., & LeWald, J. E. (1970) *Acta Endocrinol. (Kobenhavn Suppl.)* **147,** 79
(6) Twomey, S. L., & Sweat, R. (1975) *Clin. Biochem.* **8,** 169

Identification and Confirmation of Pyrantel- and Morantel-Related Residues in Liver by Gas Chromatography-Mass Spectrometry with Selected Ion Monitoring

MARTIN J. LYNCH and S. RICHARD BARTOLUCCI
Drug Metabolism, Central Research, Pfizer Inc., Groton, CT 06340

A gas chromatographic-mass spectrometric method with selected ion monitoring (GC/MS-SIM) is described for identification of pyrantel- and morantel-related residues in swine and beef liver. Alkaline hydrolysis of tissue liberates and converts pyrantel- and morantel-related residues to 3-(2-thienyl)-acrylic acid (TAA) and 3-(3-methyl-2-thienyl)-acrylic acid (MTAA), respectively. These thienyl acrylic acids are recovered by ion exclusion chromatography and converted to methyl ester derivatives (TAAE and MTAAE) for identification by GC/MS-SIM. When the relative intensities of the molecular ion, the base peak, and a third significant ion of the sample correspond to ion intensities of the preformed or processed standards, the identity of the residue is confirmed. The method was validated by analysis of swine and beef liver samples containing incurred residues of pyrantel and morantel under withdrawal conditions.

Pyrantel and its analog morantel are effective anthelmintic agents that belong to a family classified as tetrahydropyrimidines (Figure 1). In the United States, pyrantel tartrate (Banminth) is approved for use in swine; it is fed continuously in feed at 0.0106% (96 g/ton) and withdrawn for 24 h before slaughter, Morantel tartrate (Rumatel) was recently approved by the Food and Drug Administration for use in cattle. Under proposed conditions of use, it is formulated in a bolus or a feed premix, and is administered to cattle in a single dose at 9.7 mg/kg. Cattle are not to be treated within 14 days of slaughter.

Radiotracer metabolism studies with morantel in cattle (J. C. Faulkner and A. G. Davidson, Pfizer Ltd., Drug Metabolism, Sandwich, UK, private communication) and pyrantel in swine (1, 2) demonstrated that each drug is rapidly metabolized to a large variety of compounds. While no single chemical represents all of the tissue residues, the mixture of metabolites present in edible products hydrolyzes in alkali to *N*-methyl-1,3-propanediamine (MAPA) and thiophene acrylic acid analogs as major and minor components, respectively, of total radioactivity. Some liver radioactivity apparently arises from the re-incorporation of small labeled fragments into natural constituents. Residues of the 2 drugs are distinguished by liberation of 3-(3-methyl-2-thienyl)-acrylic acid (MTAA) from morantel and 3-(2-thienyl)-acrylic acid (TAA) from pyrantel as low level components of tissues (Figure 1). Hence, MAPA, MTAA, and TAA constitute marker compounds for these drugs (3).

Chemical assay residue depletion studies have been performed for major residues of morantel in cattle (4) and pyrantel in swine (5), using a procedure based on determination of the major fragment MAPA in tissue hydrolysates. Liver was identified as the rate-limiting tissue for depletion of residues after withdrawal of morantel and pyrantel from their respective use species under field trial conditions. When assayed as the hydrolysis product MAPA, pyrantel residues of 10 ppm or less in swine liver, and morantel residues of 0.5 ppm or less in bovine liver are required for clearance. However, assays for this

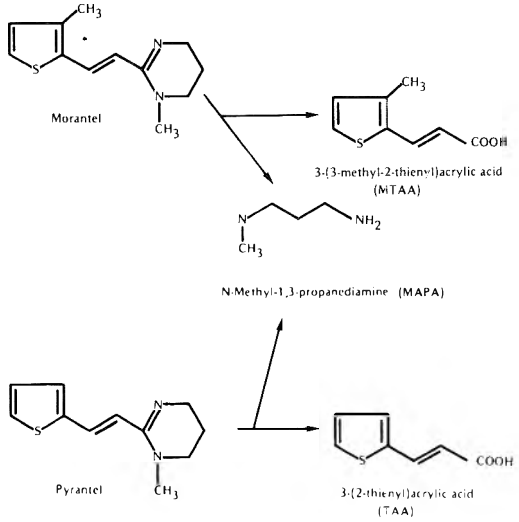


Figure 1. Alkaline hydrolysis of morantel and pyrantel.

Received October 27, 1981. Accepted December 4, 1981.

0004-5756/82/6503-0640-07$01.00

hydrolysis product do not identify the parent drug even though the use of these drugs is species-specific in the United States. Hence, this difference in required sensitivity levels for the hydrolysis product MAPA and the absence of complete specificity in the determinative assay required development of an identification procedure.

The analytical problem of identifying residues of morantel and pyrantel was solved by developing a specific chemical method for the minor fragments MTAA and TAA. This method is based on application of the technique of combined gas chromatography–mass spectrometry with selected ion monitoring (GC/MS-SIM), which has been utilized in other methods (6–9), for identification of MTAA and TAA as their methyl ester derivatives.

This report is a description of this confirmatory identification method and its application to the analysis of liver samples containing physiologically incorporated residues of morantel and pyrantel near their respective tolerances.

METHOD

Principle

Morantel- and pyrantel-related residues in liver are converted to thienyl acrylic acid homologs by alkaline hydrolysis. These marker compounds, MTAA and TAA, are separated from major interfering tissue components by ion exclusion chromatography and then are concentrated by extraction with $CHCl_3$ under acid conditions. Esterification with methanolic H_2SO_4 and extraction of their methyl ester derivatives, MTAAE and TAAE, with hexane provide further separation and concentration. Each compound is identified by monitoring the relative intensities of 3 major ions, using GC/MS-SIM. The ions monitored are the molecular ion, the base peak, and a third significant ion for the respective compounds.

Reagents

(a) *Solvents.*—Glass-distilled, or equivalent. No special precautions taken with glassware.

(b) *Chemicals.*—Reagent grade.

(c) *Ion exclusion resin.*—Macroporous, strong acid cation exchanger, AG MP-50, 100–200 mesh (Bio-Rad Laboratories, Richmond, CA 94804).

(d) *Analytical standards.*—MTAA, TAA, MTAAE, and TAAE. Provided by Pfizer Inc., Central Research, Groton, CT 06340.

(e) *Methanol–H_2SO_4 reagent (97 + 3).*—Dilute 3.0 mL concentrated H_2SO_4 to 100 mL with methanol that was dried over anhydrous Na_2SO_4. Prepare daily and use ice bath.

(f) *Standard solutions.*—(*1*) *MTAAE (3-(3-methyl-2-thienyl)-acrylic acid methyl ester).*—1 μg/mL. Dissolve 10.0 mg MTAAE in 100 mL hexane. Dilute 1.0 mL of this solution to 100 mL with hexane.

(*2*) *TAAE (3-(2-thienyl)-acrylic acid methyl ester).*—10 μg/mL. Dissolve 1.0 mg TAAE in 100 mL hexane.

(*3*) *MTAA.*—Dissolve 2.50 mg MTAA in methanol and dilute to 100 mL (concentration 25.0 μg/mL). Dilute 1.0 mL of this solution to 100 mL with methanol (2.50 μg/mL).

(*4*) *TAA.*—Dissolve 5.00 mg TAA in methanol and dilute to 100 mL (concentration 50.0 μg/mL). Dilute 2.5 mL of this solution to 10.0 mL with methanol (12.5 μg/mL).

Apparatus

(a) *GC/MS-SIM system.*—Finnigan Model 3200 quadrupole mass spectrometer equipped with Promin unit for monitoring selected ions in conjunction with Model 9500 gas chromatograph. Helium carrier gas, 34 lb/sq in; ionization energy, 70 eV; emission current, 0.74 ma. Vacuum diverter was used to vent effluent. Ion chromatograms were recorded on multi-pen Houston recorder at chart speed of 1 cm/min.

For analysis of pyrantel residues as TAAE: Pack 4 ft × 2 mm id glass column with 3% Silar 10C on 80–100 mesh Gas-Chrom Q. Operate injector, column oven, separator oven, and transfer line at 225, 175, 220 and 245°C, respectively. Approximate retention time of TAAE was 1 min.

For analysis of morantel residues as MTAAE: Pack 3 ft × 2 mm id glass column with 3% OV-17 on 100–120 mesh Chromosorb Q. Operate injector, column oven, separator oven, and transfer line at 190, 150, 220, and 190°C, respectively. Approximate retention time of MTAAE was 1 min.

(b) *Chromatographic columns.*—25 cm × 10.5 mm id, with Teflon stopcocks and 200 mL reservoir capacity (No. JC-1506, SGA Scientific Inc., Bloomfield, NJ 07003).

Preparation of AG MP-50 Resin

Transfer 100 g AG MP-50 resin to Buchner funnel equipped with coarse fritted disk and filter flask. Wash resin in sequence with 1 L methanol, 1 L water, and 500 mL 1N HCl. Stir resin with glass rod and use suction to maintain moderate filtration rate. Store filter cake in capped amber jar.

Preparation of Ion Exclusion Columns

Mix ca 7.0 g washed AG MP-50 resin in 1N HCl and transfer to 10.5 mm id glass column containing small glass wool plug to retain resin. Pack resin to height of 4 in., using glass rod, and cap resin bed with glass wool plug. Maintain level of 1N HCl slightly above resin bed.

Procedure

Dissolution and hydrolysis.—Transfer 5.0 g freshly sliced frozen tissue to 50 mL centrifuge tube. Pipet 10.0 mL 3M NaOH into tube, stopper lightly and place in a preheated silicone oil bath at 95–100°C for 16–22 h. (Note: Level of silicone oil bath should exceed that of tissue sample.) Cool hydrolysate in an ice bath, acidify (pH <1) with 4.0 mL concentrated HCl, and mix thoroughly. To validate detection of morantel- and pyrantel-related residues at levels consistent with regulatory requirements, fortify 5 g liver with 250 ng MTAA or 1.25 μg TAA. Add alkali and proceed as directed above.

Ion exclusion chromatography.—Attach powder funnel containing rapid filtering, fluted filter paper to top of an ion exclusion column, prepared as described above. Decant hydrolysate through filter paper and drain filtrate to resin. Wash residue of tissue hydrolysate in digestion tube through filter bed with 10 mL 1N HCl. Remove filter, wash resin with four 10 mL portions of 1N HCl, and drain washes to top of resin bed. Discard these effluents. Elute column with 75 mL 10% methanol in water and collect eluate in 250 mL separatory funnel. Column may be run dry in this step.

Concentration of TAA or MTAA acid eluates.—Add 1.0 mL concentrated HCl to eluate and extract with two 50 mL portions of $CHCl_3$. Collect extracts in 250 mL round-bottom flask and evaporate to dryness on rotary evaporator at 45–50°C. Transfer residue to 15 mL centrifuge tube by washing flask with 3 small portions (ca 1.0 mL each) of methanol. Place tube in 55°C hot water bath and evaporate solvent to dryness under stream of nitrogen. Esterify this residue as indicated below.

Esterification of TAA or MTAA.—Reconstitute residue with 0.2 mL freshly prepared methanol–sulfuric acid (97 + 3). Stopper and heat 1 hr in 50–55°C water bath. Remove tube from water bath, add 0.2 mL hexane to the tepid esterification solution, and thoroughly mix on test tube mixer. Follow with addition of 1.0 mL water, mix thoroughly, and centrifuge to clarify. With aid of disposable Pasteur pipet, remove most of aqueous layer. Process hexane extract by GC/MS-SIM. (Note: Hexane extracts are stable for at least 1 month when stored at reduced temperature to prevent losses through evaporation.)

Preparation of process standards for GC/MS-SIM analysis.—Pipet 0.1 mL working standard solution of TAA (12.5 μg/mL) or MTAA (2.50 μg/mL) into 15 mL centrifuge tube and evaporate to dryness at 55°C under stream of nitrogen. Add 0.2 mL methanol–sulfuric acid (97 + 3), stopper, and incubate 1 h at 55°C. As indicated in esterification procedure, add 0.2 mL *n*-hexane to esterification solution, mix, add 1.0 mL water, and centrifuge. Examine hexane layer by GC/MS-SIM. Prepare standards in triplicate.

Gas chromatography–mass spectrometry.—Set mass spectrometer to detect ion current profiles at m/z = 168, 137, and 109 for TAAE, or 182, 151, and 122 for MTAAE via Promim units. Inject 5 ng preformed TAAE or MTAAE into gas chromatograph to determine their retention times and to evaluate response of GC/MS detector. Optimum ion source voltages were adjusted for maximum sensitivity consistent with good peak shape. Following this tuning procedure, injection of neat hexane should give no response at ions of interest. Follow this with microliter injections of process standard, control, and sample tissue extracts. Allow 10 min between sample injections to clear instrument of background peaks. Measure peak height (mm) of samples and standards for each ion current profile. Normalize results with respect to intensity of base peak at m/z = 137 for TAAE and m/z = 151 for MTAAE.

Results and Discussion

Preliminary studies were conducted to optimize conditions for the hydrolysis, isolation, derivatization, and analysis of pyrantel- and morantel-related residues in tissue, using information obtained from radiotracer metabolism studies.

An overnight hydrolysis of tissue in 2 to 4 molar NaOH is required to convert pyrantel and morantel to their respective thienyl acrylic acid products. The maximum yield and recovery of these products following alkaline hydrolysis is about 75%. Prolonged hydrolysis or performing this reaction under nitrogen did not improve the yield.

The conversion of pyrantel- and morantel-related residues to thienyl acrylic acid products in the presence of tissue also converts naturally

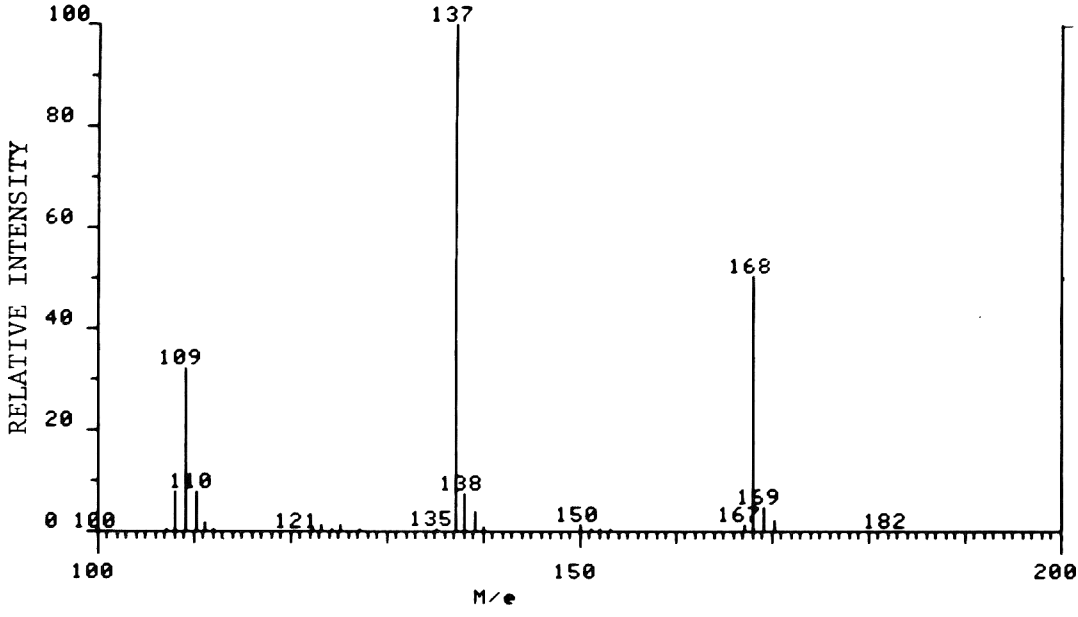


Figure 2. Mass spectrum of 3-(2-thienyl)-acrylic acid methyl ester (TAAE).

occurring materials to potential interfering materials. The bulk of these are removed by an ion exclusion process that has been described for residues of carbadox (10). Like other aromatic and aliphatic carboxylic acids, TAA and MTAA in their un-ionized forms are sorbed by the matrix of the resin polymer and excluded as the dissociated species when the pH of the eluting solvent is raised above the pKa of the organic acids. Several examples of this technique have

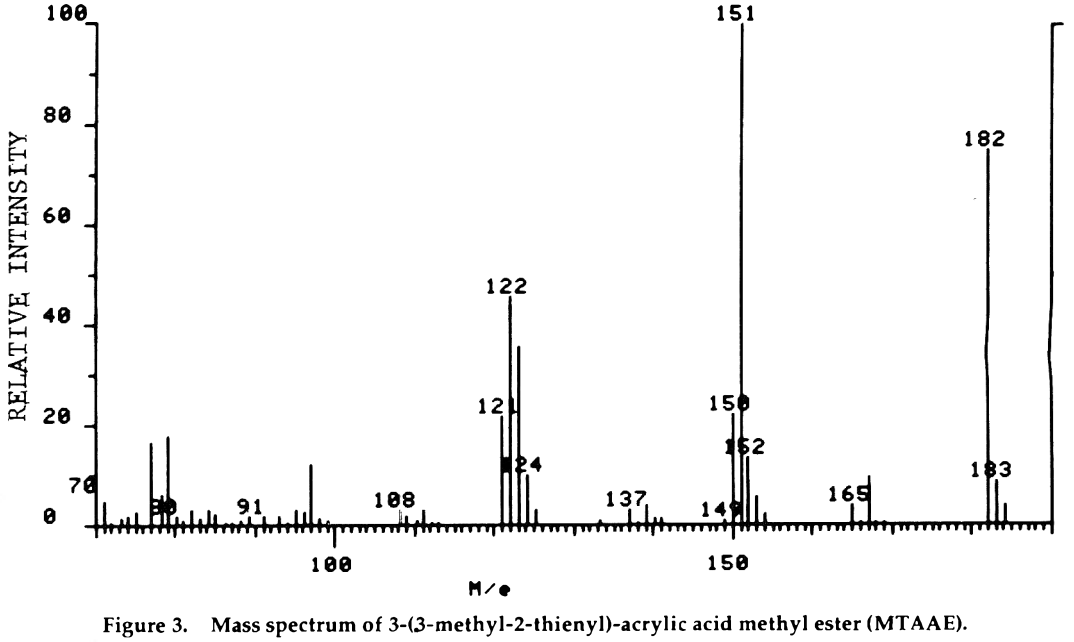


Figure 3. Mass spectrum of 3-(3-methyl-2-thienyl)-acrylic acid methyl ester (MTAAE).

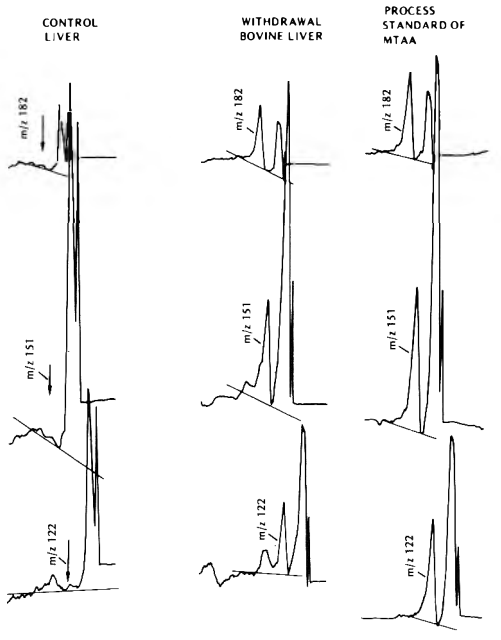


Figure 4. GC/MS-SIM analysis of withdrawal bovine liver samples for incurred residues of morantel as MTAAE. Pens were offset to monitor ions at 182, 151, and 122.

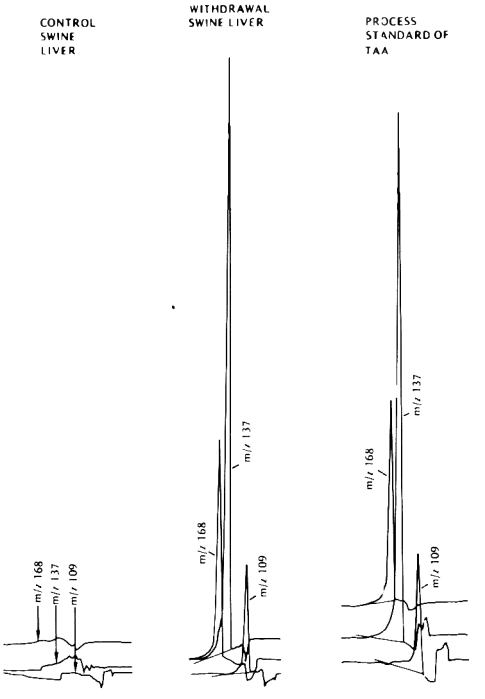


Figure 5. GC/MS-SIM analysis of withdrawal swine liver for incurred pyrantel residues as TAAE. Pens were offset to monitor ions at 168, 137, and 109.

been reported for organic acids (11–15). In the application described here, an organic modifier, methanol, is added to the eluting solvent to expedite the recovery of TAA and MTAA. Among strong cation exchange resins examined, the macroporous resin AG MP-50 was selected because of its nonswelling properties and its apparently greater sorptive capacity for nonelectrolytes. Under the conditions required for assay of withdrawal samples, 250 ng of TAA or 1.25 μg of MTAA is recovered in the ion exclusion chromatographic and subsequent extraction steps with >80% efficiency. Among several esterification procedures examined (16), sulfuric acid catalysis with dried methanol provided the best yields, convenience, ease of extraction, and compatibility with the GC/MS system. The esterification of TAA to TAAE or MTAA to MTAAE is complete under the recommended conditions for derivatization. The facile extractability of each ester into small volumes of hexane from the derivatization medium also provides a simple method for concentrating the sample.

TAAE and MTAAE are well separated according to the gas chromatographic conditions recommended for analysis. Selected ion monitoring also provides additional specificity. No interference was detected when 100 ng of TAAE or MTAAE was injected and analyzed for the homolog ester.

The overall yield of TAA from pyrantel or MTAA from morantel is about 50% of theory, and is attributed to yields of 75, 85, and 80% in the hydrolysis, ion exclusion, and esterification steps, respectively.

The mass spectra of TAAE and MTAAE under electron impact ionization conditions at 70 eV are presented in Figures 2 and 3. The spectrum of TAAE is characterized by prominent ions above m/z 100 at 109, 137 (base peak), and 168 (molecular ion), and the relative intensities of these ions are monitored. The spectrum of MTAAE is characterized by prominent ions 14 units greater than those of TAAE. Thus, the molecular ion at m/z 182, the base peak at 151, and the 122 ion in the 121–123 cluster were chosen for selected ion monitoring of MTAAE. A fragmentation pattern consisting of the sequential loss of methoxyl and carbonyl groups from the molecular ion is indicated for TAAE and MTAAE, thus accounting for the ions indicated.

The procedure described here was evaluated by assaying control and homogenized with-

Table 1. GC/MS-SIM analysis of bovine liver withdrawal samples containing incurred residues of morantel

Standard or sample identity	No. of replicates	Intensity ratios[a] 182	151	122
Trial 1				
Preformed MTAAE	3	0.59 ± 0.00	1	0.70 ± 0.02
MTAA converted to MTAAE (process stds)	3	0.60 ± 0.02	1	0.71 ± 0.01
Withdrawal liver No. 1	6	0.56 ± 0.03	1	0.68 ± 0.03
Trial 2				
Preformed MTAAE	3	0.53 ± 0.01	1	0.71 ± 0.01
MTAA converted to MTAAE (process stds)	3	0.50 ± 0.01	1	0.71 ± 0.03
Withdrawal liver No. 2	6	0.52 ± 0.02	1	0.66 ± 0.02
Trial 3				
MTAA converted to MTAAE (process stds)	3	0.51 ± 0.02	1	0.68 ± 0.03
Withdrawal liver No. 3	3	0.52 ± 0.01	1	0.64 ± 0.03

[a] Mean ± SD.

drawal liver specimens containing physiologically incorporated residues of morantel and pyrantel under withdrawal conditions. Three homogenized specimens (2–4 kg) were obtained from cattle that were sacrificed 4–6 days after receiving a bolus of morantel tartrate at the recommended use level (9.7 mg/kg). A specimen (0.3 kg) containing pyrantel-related residues was obtained from a pig that was maintained on pyrantel tartrate in feed at 96 g/ton for 117 days and sacrificed on day of withdrawal. Determinative assays for the major hydrolysis product, MAPA, indicated that these liver samples contained residues near tolerance, i.e., 0.5, 0.6, and 0.8 ppm of morantel- and 9.6 ppm of pyrantel-related residues. Hence these specimens were suitable for evaluation by the identification procedure. Under these conditions, 0.25 and 0.05 ppm of TAA and MTAA are detected in hydrolysates of swine and bovine liver, respectively. Three trials were conducted for morantel and one for pyrantel. Representative chromatograms for the identification of incurred residues of morantel and pyrantel are presented in Figures 4 and 5. These analyses (Tables 1 and 2) demonstrated that within each trial, peak height ratios of MTAAE or TAAE in the withdrawal samples corresponded to within ±10% of ion intensities of the processed and preformed standards. No major interference was found in control liver. Control swine liver supplemented with 2 ppm of TAA and assayed for morantel-related residues as MTAAE shows no interference. The identity of TAAE was readily confirmed in this sample, indicating that pyrantel residues may not be identified as morantel residues by this procedure.

Based on these results, the GC/MS-SIM procedure is suitable for verifying the identity of pyrantel or morantel residues in liver at their respective tolerances.

Acknowledgment

The authors are grateful to Norman Glidden for preparation of samples and Elbridge Luther for obtaining mass spectral data.

Table 2. GC/MS-SIM analysis of swine liver containing incurred residues of pyrantel

Standard or sample identity	No. of replicates	Intensity ratios[a] 168	137	109
Preformed TAAE	3	0.40 ± 0.01	1	0.23 ± 0.01
TAA converted to TAAE (process stds)	3	0.39 ± 0.01	1	0.22 ± 0.01
Withdrawal liver	8	0.38 ± 0.01	1	0.20 ± 0.01

[a] Mean ± SD.

REFERENCES

(1) Faulkner, J. K., et al. (1972) *J. Sci. Food. Agric.* **23,** 79–91
(2) Figdor, S. K., Schach von Wittenau, M., & Lynch, M. J. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1228–1231
(3) Perez, M. K. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1183–1191
(4) Lynch, M. J., & Bartolucci, S. R. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 227–233
(5) *Food Additives Analytical Manual* (1973) Food and Drug Administration, Washington, DC
(6) Cala, P. C., et al. (1972) *J. Agric. Food Chem.* **20,** 337–340
(7) Day, E. W., Jr, Varratta, L. E., & Sieck, R. F. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 520–524
(8) Ryan, J. J., & Pilon J.-C. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 817–820
(9) VandenHeuvel, W. J. A., III, Wood, J. A., DiGiovanni, M., & Walker, R. W. (1977) *J. Agric. Food Chem.* **25,** 386–389
(10) Lynch, M. J., & Bartolucci, S. R. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 66–70
(11) Harlow, G. A., & Morman, D. H. (1964) *Anal. Chem.* **36,** 2438–2442
(12) Goodman, G. W., Lewis, B. C., & Taylor, A. F. (1969) *Talanta,* **16,** 807–812
(13) Lehotay, J., & Traiter, M. (1974) *J. Chromatogr.* **91,** 261–266
(14) Hanai, T., Walton, H. F., Navratil, J. D., & Warren, D. (1978) *J. Chromatogr.* **155,** 261–271
(15) Tanaka, K., Ishizuka, T., & Sunahara, H. (1979) *J. Chromatogr.* **174,** 153–157
(16) Nicholson, J. D. (1978) *Analyst,* **103,** 1–28

METALS AND OTHER ELEMENTS

Dry Ashing, Hydride Generation Atomic Absorption Spectrometric Determination of Arsenic and Selenium in Foods

GERARD K. H. TAM and GLADYS LACROIX
Health and Welfare Canada, Food Research Division, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario, Canada K1A 0L2

A dry ashing, flameless atomic absorption spectrometric method was evaluated to determine arsenic and selenium in foods. Samples were dry-ashed with $Mg(NO_3)_2$-MgO and dissolved in HCl. Selenate was reduced to selenite by boiling in 4N HCl, and arsenate to arsenite by treatment with KI. Hydrides of arsenic and selenium were generated by the addition of $NaBH_4$ and were swept by nitrogen and hydrogen into a thermally heated silicate tube furnace. The detection limit was about 5 ppb for each element based on a 10 g sample. Analytical results obtained for several samples of NBS reference materials agreed with the certified values. The procedure was evaluated by another laboratory and results were satisfactory.

Trace levels of arsenic and selenium have been determined in foods by hydride generation atomic absorption spectrometry (1–4). A collaborative study conducted by Holak (4) on a closed system digestion technique and a specified hydride generator showed good agreement among the 7 participating laboratories. However, another collaborative study by 28 laboratories, which specified a HNO_3-$HClO_4$-H_2SO_4 digestion procedure but allowed the free choice of hydride generation and spectrometric instrumentation, was reported by Ihnat and coworkers (5, 6). Their results showed poor within- and among-laboratory precision, although results averaged over all laboratories agreed reasonably well with reference values. They recommended reassessment of acid digestion and dry ashing procedures for dissolution of biological materials.

Several dry ashing procedures (7–9) used to decompose biological samples, including marine samples, gave good recoveries of As. One of the dry ashing procedures (9) also gave good recovery of Se in plant and animal tissue (10). A survey of the literature indicates that As has been determined by atomic absorption spectrometry (AAS) after dry ashing to decompose the sample (3, 11, 12), but no similar study on Se has been reported. The present study evaluates the procedure for determination of both As and Se in food products, using the established dry ashing technique (9, 10) followed by hydride generation AAS. The procedure was studied in 2 laboratories using different hydride generation systems.

Received August 24, 1981. Accepted November 6, 1981.

Experimental

Apparatus

Varian Techtron AA 120 spectrometer, Varian Model 64 vapor generation accessory, and Vycor tube furnace were used in the authors' laboratory. Detailed description is given in ref. 11.

A second laboratory used a Perkin-Elmer AA 360 spectrometer equipped with D_2 arc background corrector, EDLs for As and Se, and MHS-1 hydride generator, set up according to manufacturer's suggestion.

Reagents

(a) *Magnesium oxide.*—Ash analytical grade $Mg(NO_3)_2 \cdot 6H_2O$ overnight in 500°C muffle furnace and grind to fine powder. This MgO will give lower blank for As and Se compared with commercial MgO.

(b) *Ashing aid.*—Dissolve 80 g $Mg(NO_3)_2 \cdot 6H_2O$ in 200 mL water. Add 8 g MgO and shake well before use.

(c) *Potassium iodide solution.*—30% w/v. Prepare in water and store in refrigerator.

(d) *Sodium borohydride solution.*—3% w/v. Dissolve 3 g $NaBH_4$ (Fisher Scientific Co.) in 100 mL 0.5% NaOH solution and filter through Millipore paper (type DA, 0.65 μm). Store in refrigerator.

(e) *Arsenic standard stock solution.*—1000 ppm. Dissolve 1.320 g As_2O_3 in minimum volume of 20% w/v NaOH solution and neutralize with HCl. Dilute to 1 L with water.

(f) *Arsenic standard working solutions.*—(1) *10.0 ppm:* Pipet 1.00 mL As standard stock solution into 100 mL volumetric flask, add 5 mL 6N HCl and dilute to volume. (2) 100 ppb: Pipet 1.00

0004-5756/82/6503-0647-04$01.00

Table 1. Effect of magnesium oxide on recovery of 50 ng As or Se [a]

Mg^{+2} concn, [b] mg/mL	As recd, [c] %	Se recd, %
0	100	100
0.6–6.0	100	100
9.0	86	87
12.0	72	80

[a] Average of duplicate analyses.
[b] Volume of solution 20 mL.
[c] Data from ref. 11.

mL of the 10.0 ppm solution into 100 mL volumetric flask and dilute to volume. Prepare fresh daily.

(g) *Selenium standard stock solution.*—1000 ppm. Dissolve 1.000 g Se in HNO_3 and dilute to 1 L with water.

(h) *Selenium standard working solutions.*—10.0 ppm and 100 ppb. Prepare as described in (f).

Sample Digestion and Dissolution

Add 10 mL ashing aid to sample (1 to 10 g) in 150 mL beaker and mix well. Cover beaker with watch glass and dry contents in 110°C oven. Transfer beaker to cold muffle furnace and slowly raise temperature of furnace to 500°C. Ash overnight according to the method of Tam and Conacher (9). Cool sample to room temperature. Add 5 mL water to wet ash and dissolve in 35 mL 6N HCl. Heat solution on hot plate almost to boiling and maintain 20–30 min to reduce selenate to selenite. Transfer to 50 mL volumetric flask and dilute to volume with 6N HCl. Prepare duplicate blanks by carrying 10 mL ashing aid solution through the procedures.

Determination

Pipet duplicate aliquots containing <100 ng As or Se into hydride generation tubes. Add 6N HCl to bring volume to 20 mL. To determine selenium, inject 3 mL 3% $NaBH_4$ solution through a syringe to generate hydrogen selenide, and measure peak height of signal. To determine As, add 1 mL 30% KI solution and let mixture stand 15–20 min before addition of $NaBH_4$. (Arsenate is reduced to arsenite by KI.)

Pipet standard solution containing 10, 20, 40, 60, 80, and 100 ng As or Se into 20 mL 6N HCl solution. Treat standards in same manner as samples. Calculate concentration of As or Se in sample, using standard calibration curve.

Interference Studies

Add various amounts of MgO, common cations and anions to a solution containing 50 ng selenite. Adjust final volume to 20 mL with 6N HCl. Generate hydrogen selenide as described above. Calculate recovery of Se through the hydride generation step.

Results and Discussion

Effect of Ashing Aid

Because $Mg(NO_3)_2$ and MgO were used for decomposition, the resulting ashed solution contained a large amount of magnesium ion. The effect of this on the recovery of As has been reported previously (11). We found a similar pattern for Se, as shown in Table 1. Quantitative recovery of As and Se was observed up to 6.0 mg/mL of Mg^{+2}. This amount of Mg^{+2} added was equivalent to the amount of Mg^{+2} in 10 mL of the dissolved ashed sample, which had a total volume of 50 mL. Recoveries of As and Se decreased with larger amounts of Mg^{+2}. Thus, we decided to limit the aliquot taken from the ashed solution to a maximum of 10 mL.

Effect of Common Ions

The effect of common cations and anions on the recovery of As has been reported in a previous study (11). The effect of these same ions on the recovery of Se is similar. No interferences on the recovery of 50 ng Se were observed in the

Table 2. Suppression effect of several ions on the recovery (%) of 50 ng selenium

Amt present, μg	Ion				
	Bi^{+3}	Sb^{+5}	Sn^{+4}	As^{+5}	As^{+3}
5	100	100	100	100	55
10	100	100	100	100	28
20	87	100	72	80	
50	80	85	60	60	
100	58	60	52	55	
200	36	50	32	50	

Table 3. Accuracy evaluation of dry ashing-AAS method by analysis of NBS Standard Reference Materials

Sample[a]	As, ppm		Se, ppm	
	Dry ashing-AAS	Certified value	Dry ashing-AAS	Certified value
NBS 1577 Bovine Liver	0.023 ± 0.012[b]	0.055 ± 0.005	1.14 ± 0.091[b]	1.1 ± 0.1
NBS 1568 Rice Flour	0.436 ± 0.018	0.41 ± 0.05	0.338 ± 0.003	0.4 ± 0.1
NBS 1571 Orchard Leaves	12.0 ± 0.38	10 ± 2	0.070 ± 0.020	0.08 ± 0.01

[a] Sample size 0.8–1.2 g.
[b] Average ± standard deviation (triplicate analyses).

Table 4. Recovery of As and Se added to NBS samples

Sample[a]	As added, ng	As recd, %	Se added, ng	Se recd, %
NBS 1577 Bovine Liver	200	105.8 ± 3.1[b,c]	200	93.0 ± 7.9[b,c]
NBS 1568 Rice Flour	200	116.6 ± 3.0	200	92.0 ± 6.2
NBS 1751 Orchard Leaves	2200	79.3 ± 12.1	200	80.6 ± 4.0

[a] Sample size 0.8–1.2 g.
[b] Average ± standard deviation (triplicate analyses).
[c] Recovery based on the results from dry ashing-AAS.

presence of up to 500 μg Na^+, K^+, Ca^{+2}, CrO_4^{-2}, $Cr_2O_7^{-2}$, MnO_4^{-1}, or PO_4^{-3}. Recovery of selenium was reduced 10% by the presence of 250 μg Zn^{+2}, Cd^{+2}, Pb^{+2}, Co^{+2}, Cu^{+2}, or Fe^{+3}. However, more significant interference on the recovery of Se was observed for Bi, Sb, Sn, and As (Table 2). A suppression effect of 13 to 28% was observed for Bi^{+3} and Sn^{+2} respectively when present in a 400-fold weight excess of these ions to selenite. The most serious suppression effect, that of As^{+3}, was fortunately not important because arsenic exists only as arsenate after dry ashing. No attempt was made to reduce or eliminate the interference effects from these ions in the present study.

Accuracy and Precision

Previous studies have shown that As and Se could be quantitatively recovered using the above dry ashing procedure (9, 10). The accuracy of the present method (dry ashing-AAS) was tested by determining both As and Se in several NBS Standard Reference Materials. Certified levels of As and Se in these samples ranged from 0.055 to 10 ppm and from 0.08 to 1.1 ppm, respectively. Table 3 shows the results of triplicate analyses. Experimental results agreed well with the certified values, with the exception of arsenic in the bovine liver sample. For this sample, the concentration of arsenic is 0.055 ppm which is approximately the detection limit for a 1 g sample. Recoveries of As and Se added to these NBS samples before dry ashing at levels of 200–2000 ng ranged from 79 to 117% as shown in Table 4.

To study the repeatability of the method, several freeze-dried powder samples (milk, chicken, and cod) were dry ashed and analyzed in quadruplicate on 2 different days. The results are shown in Table 5. The coefficients of variation ranged from 3 to 22% and 1.6 to 16% for As and Se, respectively. They were significantly higher at low concentrations near the detection limits (50 ppb for a 1 g sample for both elements, based on 3 times standard deviation of the blanks). At higher concentrations, the coefficients of variation were approximately 5% or less.

Evaluation of the Method

The described method was evaluated in the authors' laboratory and in another regional laboratory of the Health Protection Branch. Five

Table 5. Repeatability study of dry ashing-AAS method[a]

Sample	As, ppm	Se, ppm
Cod	9.07 ± 3.0[b]	5.65 ± 4.6[b]
Chicken	0.371 ± 5.9	0.746 ± 1.6
Milk powder	0.081 ± 22.2	0.128 ± 16.4

[a] Samples were individually dry ashed and analyzed in quadruplicate on 2 days.
[b] Average ± coefficient of variation (%).

Table 6. Determination of As and Se in check samples in 2 laboratories [a]

	As, ppb		Se, ppb	
Sample [b]	Lab. A	Lab. B	Lab. A	Lab. B
Apple powder	<50	<50	<50	<50
Milk powder	61, 79	<50	92, 82	102, 124
Chicken powder	65, 64	56, 57	620, 698	613, 660
Liver powder	362, 403	350, 384	940, 1008	866, 815
Cod powder	8756, 9323	9450, 8710	1137, 1213	1085, 1204
Lobster paste	5030, 5065	4900, 4720	1050, 1085	868, 1055
Ham	<10	<10	148, 155	146, 149
Beets	<10	<10	<10	<10
Apple sauce	<10	<10	<10	<10
Apple juice [c]	156, 170	180, 187	<10	<10

[a] Duplicate analyses.
[b] Sample size: 0.8 to 1.2 g for powder, 2 to 10 g for others.
[c] Sample spiked with 150 ppb As.

samples were prepared by each laboratory and portions were sent to the other laboratory. Each laboratory analyzed the 10 samples in duplicate for both As and Se. The dry ashing procedure was strictly followed, but the hydride generation systems were different as mentioned in the experimental procedure. Table 6 shows that the results for As and Se agree well. Recoveries of As and Se added to the samples ranged from 80 to 117%.

After completion of the study, an article by Reamer and Veillon was published (13). They showed that Se could be determined accurately by hydride generation AAS after decomposition of the sample by a very similar dry ashing procedure. The present study agrees very well with their results.

In conclusion, the described method provides a convenient and accurate means for determining As and Se, using a specified dry ashing procedure and 2 commercially available hydride generators. It avoids the use of $HClO_4$ which requires a specific fume hood for safety precautions. The disadvantage is that the sample size is limited to 10 g (1 g for dry powder) because of increased losses of fine ashes with a large sample. This method is currently being used to survey the levels of both elements in food products in Canada.

Acknowledgments

The authors thank G. Dube and R. Crocker of the Halifax regional laboratory for their participation in the check sample determinations.

References

(1) Fiorino, J. A., Jones, J. W., & Caper, S. G. (1976) *Anal. Chem.* **48,** 120-125
(2) Ihnat, M., & Miller, H. J. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 813-825
(3) Evans, W. H., Jackson, F. J., & Dellar, D. (1979) *Analyst* **104,** 16-34
(4) Holak, W. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 485-495
(5) Ihnat, M., & Miller, H. J. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1414-1433
(6) Ihnat, M., & Thompson, B. K. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 814-839
(7) LeBlanc, P. J., & Jackson, A. L. (1973) *J. Assoc. Off. Anal. Chem.* **56,** 383-386
(8) George, G. M., Frahm, L. J., & McDonnell, J. P. (1973) *J. Assoc. Off. Anal. Chem.* **56,** 793-797
(9) Tam, G. K. H., & Conacher, H. B. S. (1977) *J. Environ. Sci. Health* **B12,** 213-227
(10) Tam, G. K. H., & Lacroix, G. (1979) *J. Environ. Sci. Health* **B14,** 515-524
(11) Tam, G. K. H., & Lacroix, G. (1980) *Int. J. Environ. Anal. Chem.* **8,** 283-290
(12) Uthe, J. F., Freeman, H. C., Johnston, J. R., & Michalik, P. (1974) *J. Assoc. Off. Anal. Chem.* **57,** 1363-1365
(13) Reamer, D. C., & Veillon, C. (1981) *Anal. Chem.* **53,** 1192-1195

PLANTS

Effects of Particle Size and Method of Milling on Determination of Morphine in *Papaver somniferum* L. Straw

BEATRIZ FERREIRA ENGELKE and PHILLIP G. VINCENT
U.S. Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Beltsville, MD 20705

Different particle sizes of blade- and ball-milled poppy straw were separated and analyzed for morphine by high pressure liquid chromatography. Morphine content varied up to 70% among the different size particles. This may account for discrepancies observed in percentages of morphine found among random aliquots. Homogenizing a carefully quartered sample of blade-milled straw by further pulverization in a percussion ball mill 2 min and taking a quartered aliquot for morphine analysis improved the reproducibility of results.

Poppy straw, the dry, ripe, non-incised deseeded capsule of *Papaver somniferum* L. harvested with 20 cm of stem attached, is an important commercial commodity (1, 2) and in some countries has replaced opium (3) as the raw material for medically important phenanthrene alkaloids. Disparity among results obtained by different laboratories and within the same laboratory for morphine determinations in milled poppy straw led to a study of morphine content in the different size particles constituting the sample (4). Blade-milled poppy straw was passed through a set of sieves of progressively decreasing mesh apertures, and the resulting different size groups of particles were analyzed separately. Results showed large differences (up to 70%) in the percentages of morphine found among the groups. The need for further pulverization of blade-milled straw became evident.

Experiments were carried out using a percussion ball mill for various lengths of time with different numbers of balls and different amounts of tissue to produce optimal particles. The problem of obtaining a representative aliquot from the original sample before subjecting it to further pulverization was solved by using the quartering technique.

Results of this study will contribute to the reproducibility of alkaloid analyses in poppy straw.

Experimental

Apparatus and Reagents

(a) *High pressure liquid chromatograph.*—Spectra-Physics, equipped with gradient system pumping and solvent programmer; 30 cm × 4 mm stainless steel columns packed with 10 μm porous silica gel (μPorasil, Waters Associates), with 5 μm steel end frit fittings. Multiple wavelength ultraviolet-visible detector (Schoeffel SF 770). On-column injections made with syringe-loading injector (Model 7120, Rheodyne) equipped with 10 μL loop.

Operating conditions: column oven temperature 25°C; solvent system *n*-hexane–methylene chloride–ethanol–diethylamine (300 + 30 + 40 + 0.5); flow rate 2.4 mL/min at 1850 psi; detection at 285 nm at 0.04 absorbance unit full scale (AUFS) (5).

(b) *Alkaloid standard solution.*—Dissolve 0.2 g morphine in 100 mL ethanol. Divide mixture into 5 mL portions and store at −10°C in tear-away sealed serum vials.

(c) *Solvents.*—Glass-distilled, reagent grade, degassed and filtered to pass 0.20 μm sieve.

(d) *Sieves for particle separation.*—U.S. Bureau of Standards, Standard Screen Series, 1919, sieve Nos. 20, 40, 60, and 80, corresponding to sieve openings of 840, 420, 250, and 177 μm, respectively.

(e) *Percussion ball-mill pulverizer.*—Prolabo, Microboyeur Quantitatif Dangoumau (Prolabo, 12 rue Pelie, 75 Paris XI, France): Mill in which matter to be pulverized is put into chamber together with steel balls. Chamber is moved vertically at 700 cycles/min. Head of mill accommodates one 150 mL or two 65 mL chambers. Each mill chamber comes with a set of steel balls, e.g., 65 mL chamber, one 20 mm ball, four 12 mm balls, or twelve 8 mm balls to be used with hard, brittle, or intermediate substances, respectively.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

Received October 15, 1981. Accepted December 14, 1981.

0004-5756/82/6503-0651-04$01.00

Extraction of Poppy Straw

Procedure is described in refs 4 and 5. Briefly, 30 mL of 5% aqueous acetic acid is added to the poppy straw and sonicated for 30 min. Resultant suspension is adjusted to pH 8.5 with concentrated NH_4OH, and 10 mL $CHCl_3$–methanol (3 + 1) is added. Mixture is agitated, and sonicated 10 min. Emulsion formed is broken by centrifugation and the aqueous phase is re-extracted twice. Organic phase containing morphine is concentrated under vacuum, reconstituted in 2 mL absolute ethanol, filtered through a Swinney syringe filter, and stored at −10°C in tear-away sealed serum vials.

Procedure

Individually mix the unevenly blade-milled samples of poppy straw, using quartering technique, i.e., place entire sample on smooth surface, pile it into a cone, cut cone into 2 equal parts, and then cut these 2 halves into 2 equal parts. Of the 4 equal parts obtained, take the 2 furthest apart. Mix them together. Repeat quartering as many times as necessary to reduce sample to ca 10 g. Take 1 g aliquots for experimentation and dry weight determination (100 mg in 104°C oven for 18 h). Place aliquots separately into 65 mL chamber of ball mill with either one 20 mm ball or six 8 mm balls and pulverize for 1, 2, 3, and 4 min. (Milling for 1 min was not sufficient to make a significant difference in the size of particles and experimentation for this time was terminated.) Take powders obtained from 2, 3, and 4 min milling, quarter them, take 250 mg aliquot, and extract for morphine determination (5). Weigh remainder of each sample and separate different size particles by sieving through set of 4 sieves. Determine weight of each size group, take an aliquot, and extract for morphine determination by HPLC (5).

Results and Discussion

Samples 12A and 15A were chosen as prototypes from among 20 samples used in our studies. Data obtained on the effect of milling time and milling procedure on the HPLC determination of morphine in poppy straw Sample 12A showed for 2-, 3-, and 4-min pulverization in a 65 mL chamber using one 20 mm ball, 0.51, 0.32, and 0.29% morphine, respectively. Using six 8 mm balls, 0.53, 0.30, and 0.31% morphine was found in samples milled 2, 3, and 4 min, respectively. These values on aliquots taken by quartering are means of 3 HPLC determinations corrected for dry weight. The standard deviation was 0.01 in all determinations on ball-milled samples. Determinations on blade-milled samples showed morphine content of 0.55%.

Results show that length of time of milling was important: 2-min pulverization was optimal for morphine recovery; 3- and 4-min pulverization showed a loss of recovery of 40 and 42% morphine in comparison with 2-min pulverization, respectively. Results also indicate that pulverization with either set of balls was equally effective. Use of six 8 mm balls was preferred because of diminished machine noise

In this particular sample, the percentage of morphine obtained from the blade-milled samples, 0.55, was 5% higher than the average of the percentages obtained from tissue that was ball-milled 2 min, which is an acceptable analytical difference. Extreme care was taken in quartering the blade-milled samples, thus diminishing the possibility of error. This has not always been the case with the different laboratories performing the analyses because there has been no agreement on sample preparation protocol. This sample had been reported as containing 0.76, 0.69, 0.55, 0.46, and 0.42% morphine in interlaboratory tests.

The amount of poppy straw subjected to ball-milling also was important. Small quantities (about 250 mg) adhered to the ball bearings and had to be removed by washing directly with extracting solution. The percentages of morphine found in these samples were smaller than percentages found when >250 mg was pulverized for the same length of time. For example, for Sample 15A we obtained 0.25, 0.37, 0.39, and 0.40% morphine for the respective size particles 840, 420, 250, and 177 μm when ball-milling <250 mg for 3 min; the weighted average was 0.35% morphine vs 0.40% found when pulverizing amounts of >250 mg for the same length of time (Table 1). Ball-milling amounts <1 g should be avoided. We believed this deterioration of morphine to be, in part, caused by increased heat of friction during ball-milling.

Table 1 shows variations in morphine determinations that are encountered for different particle sizes of poppy straw tissue because of sites of biosynthesis and accumulation of alkaloids are located in tissues of different consistencies, e.g., laticifers are much finer cell types than are sclerenchyma. This laboratory cooperated in an electron microscopy study with M. E. Zavala which identified cellular and subcellular sites of alkaloid synthesis and localization in *P. somniferum* by using radio-labeled alkaloids (^{3}H-morphine, ^{3}H-salutaridine, and ^{14}C-reticu-

Table 1. HPLC determination of morphine in sieved particles of pulverized poppy straw (Sample 15A) [a] prepared by blade- and ball-milling

Size of sieved particles, μm	Blade-milled			Ball-milled 2 min			Ball-milled 3 min		
	g Particles/100 g	Morphine, %	Morphine, [b] g	g Particles/100 g	Morphine, %	Morphine, g	g Particles/100 g	Morphine, %	Morphine, g
>840	4.5	0.55	0.025	ND [c]	ND	ND	ND	ND	ND
840	60.5	0.47	0.284	18.5	0.45	0.083	4.82	0.23	0.011
420	15.2	0.44	0.067	31.2	0.47	0.147	63.5	0.38	0.241
250	6.6	0.43	0.028	17.4	0.49	0.083	13.3	0.45	0.059
≤177	13.2	0.35	0.046	32.4	0.43	0.139	18.46	0.54	0.099
		$\bar{X}$ = 0.45	Σ = 0.45		$\bar{X}$ = 0.46	Σ = 0.45		$\bar{X}$ = 0.40	Σ = 0.41
Max. Δ% [d]		57			14			135	

[a] Aliquots taken by quartering. Results are the mean of 3 determinations corrected for dry weight.
[b] Proportion in which the morphine is distributed of the different groups of particles or weighted average.
[c] Not determined. This fraction was reduced on milling and passed to the next sieve.
[d] Δ% = (x-y/y) × 100.

line) (M. E. Zavala and P. G. Mahlerg, Indiana University, Department of Biology, Bloomington, IN (1980) private communication). Results indicated that the labeled alkaloid uptake and accumulation was restricted to the vascular bundles in the mature organs of *P. somniferum*. All sizes of vascular bundles have laticifers that lie between the xylem and phloem. Silver grains from radioactive decay of ^{14}C- or ^{3}H-precursors were deposited over laticifers, xylem, and vascular parenchyma. They were not found over mesophyll or epidermal cells. Because our milled plant tissue contained all these tissues, it was imperative to homogenize the sample to obtain a representative specimen.

Table 1 shows the variation in morphine content among the different size groups of particles, the percentage of each group by weight, and the morphine present in each size group. These last values were useful in ascertaining the true content of morphine in unsieved samples, their sum being the weighted average of morphine in the sample.

Our research shows the importance of taking aliquots by quartering, because if an aliquot taken at random happens to contain a disproportionate share of a given particle size, high or low in morphine as the case might be, the resulting analysis will not reflect the real value of morphine in the sample. This may have adverse economic consequences either for the buyer or the producing country. The problem of the accuracy of analysis of poppy straw has become more pressing because the trend in the international legal narcotic market is toward harvesting poppy straw instead of opium to prevent diversion of the latter to the illicit traffic (1–3).

Table 1 shows that pulverizing in a ball mill for 2 min improves the probability of taking a homogeneous aliquot; the percentage of morphine does not vary so widely among the different size particles as it does in the blade-milled samples. Table 1 shows that particles obtained by blade-milling have a maximum difference of 57% among themselves (max. Δ%), whereas the 2-min ball-milled particles have a maximum difference of only 14%. The percentages by weight of the particles are also more evenly distributed. The advantage of a finer particle size is that it offers a greater surface area for solvent extraction.

The reproducibility of results using the method outlined here was tested by selecting some of the samples that had previously shown, in both inter- and intralaboratory studies, large discrepancies among reported results of morphine analysis. The samples were spiked with $^{14}CH_3$-*N*-codeine, an aliquot was taken by quartering and was ball-milled for 2 min, and then quartered again. This last aliquot was extracted and the morphine content was determined (5). Using sample 18A as an example, we obtained a value of 0.45% and 0.43% morphine (<5% difference) for 2 replicates and recovered 98.86% of the radioactive codeine. This sample had been reported by the different laboratories as having from 0.47 to 0.28% of morphine. Thus, sample preparation proved to be the determining factor in the reproducibility of results, all other factors being equal.

Summary and Conclusions

Preparation of poppy straw to obtain a homogeneous sample is as critical to quantitative

analysis of its morphine content as are the subsequent analytical procedures. We recommend preparing samples as follows: Quarter the dry poppy straw to obtain a representative sample of about 1 g. Ball-mill for 2 min with six 8 mm balls. Quarter again, weigh 250 mg aliquot, and extract for morphine. Correct for dry weight. This laboratory used an HPLC method but that does not preclude the use of other procedures for morphine analysis.

Acknowledgments

The authors thank Olav Braenden, Director Emeritus of the United Nations Narcotic Laboratory, Vienna, Austria, for recommending the acquisition of the percussion micro mill "Dangoumau" and for allowing them to draw on the experience acquired by the U.N. Laboratory on processing hundreds of samples of poppy straw for distribution to laboratories throughout the world.

REFERENCES

(1) Ludwicki, H. (1951) *Acta Pol. Pharm.* **8**, 97–120
(2) United Nations (1974) *Statistics on Narcotic Drugs for 1974*, E/INCB/31, "Poppy straw and concentrate of poppy straw," International Narcotics Control Board, Geneva, Switzerland, p. VIII
(3) United Nations (1978) *Report of the International Narcotics Control Board*, E/INCB/41, Geneva, Switzerland, "Turkey," pp. 11, 23; "Morphine manufactured from poppy straw," p. 40
(4) Vincent, P. G., Gentner, W. A., Kuppers, F J. E. M., & Salemink, C. A. (1979) *J. Pharm. Sci* **68**, 87–89
(5) Vincent, P. G., & Engelke, B. F. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 310–314

FERTILIZERS

Factors Affecting the Determination of Citrate-Insoluble Phosphorus

FRANK J. JOHNSON and JAMES A. FARLEY
Tennessee Valley Authority, National Fertilizer Development Center, Division of Chemical Development, Muscle Shoals, AL 35660

Interlaboratory imprecision of results for citrate-insoluble P_2O_5 is greater than desired, especially for analyses of triple superphosphate (TSP). A systematic study of the method indicates that the most significant contributor to the imprecision is the fineness of the ground sample. Other factors affecting results are sample weight, bath temperature, and extraction time. Comparable results are reported for TSP analyzed by the AOAC official method on samples prepared by grinding to −20 + 200 mesh, on unground samples, and on samples disintegrated by an ultrasonic bath. Similar tests on diammonium phosphate, using the same 3 sample preparation techniques, yield results that are not comparable.

The interlaboratory variation in the determination of citrate-insoluble phosphorus (CI P_2O_5) by the AOAC official final action method (1) is greater than expected. For example, on 1980 Magruder samples, the average high value reported for CI P_2O_5 was 44% greater than the grand average, and the average low value reported was 43% below the grand average. Values of 31 and 37% were obtained when the same observations were made in the Association of Florida Phosphate Chemists (AFPC) check sample program.

Two recent sudies were concerned with CI P_2O_5 in diammonium phosphate (DAP). One of these studies, carried out at the Tennessee Valley Authority (TVA) (2), concluded that the use of marginal-quality phosphate rock and increased use of sludge from phosphoric acid have increased the quantity of CI P_2O_5 in DAP. The other study, conducted by the AFPC (R. J. Hirko (1980) AFPC, unpublished report), systematically studied the many (somewhat loosely defined) steps in the official method for determination of CI P_2O_5. The TVA study identified some reasons why DAP currently being produced may have a greater CI content, but it did not address variations in its determination. The study by AFPC attempted to separate the contribution of each step in the CI procedure to the final result, but their findings were inconclusive.

The purpose of our study was to examine the several factors that may influence the results obtained by the CI P_2O_5 method and to make appropriate recommendations.

METHOD

Citrate-insoluble phosphorus.—**2.044-2.046(a)** with noted modifications.

Discussion

Because the flushing action of the water wash and the bathing action of the citrate extraction may both depend on particle size of the sample, this was the first parameter investigated in the search to find the cause of imprecision in the CI P_2O_5 method. Five samples of triple superphosphate (TSP) from different manufacturers and different regions of the country were selected for study. About 250 g of each sample was ground in a Mikro-samplmill fitted with 1 mm screen. Each ground sample was screened, and the size fractions are tabulated in Table 1. The distributions of size fractions were surprisingly similar, even though the unground samples were significantly different in physical appearance.

Sample 1 was selected for analysis to see if the chemical composition of the size fractions was different. Size fractions smaller than 100 mesh were combined, and the samples were analyzed for the major constituents. The data in Table 2 show that the chemical composition is not different for the 3 fractions.

CI P_2O_5 was determined in each of the fractions from the 5 samples. These data are shown in Table 3. In addition, an unground portion of each sample was analyzed for CI P_2O_5 and 3 samples were analyzed for CI P_2O_5 after disintegration in an ultrasonic bath. These data are also shown in Table 3. Although there was some variation, in general, the data show consistency for the 3 larger screen sizes and significantly lower values for the 2 smallest screen sizes. Sample 5 was an exception to the norm; it gave essentially the same result for all screen sizes.

Received October 27, 1981. Accepted December 28, 1981. This paper was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981, at Washington, DC.
F. J. Johnson is the Associate Referee on Phosphorus.

0004-5756/82/6503-0655-04$01.00

Table 1. Size fractions (% of total) of ground triple superphosphate samples

Sample	Screen fraction				
	−20 +40[a]	−40 +100	−100 +200	−200 +325	−325
1	2.4	48.3	29.4	16.4	3.5
2	1.8	49.1	30.0	16.6	2.5
3	2.1	49.7	30.1	16.7	1.4
4	2.7	47.0	27.7	18.9	3.7
5	2.4	44.1	26.6	19.2	7.7

[a] Passed No. 20 screen, retained on No. 40 screen.

Because the values obtained on the −20 + 200 mesh fractions were more consistent and more nearly matched the values obtained on the unground samples and by ultrasonic disintegration, this fraction was used in all succeeding work. The extreme difference found among the screen fractions may explain the wide differences found in some interlaboratory comparisons for CI P_2O_5.

The experiment on size fractions of TSP was repeated using 3 DAP samples that had significant CI P_2O_5. Little or no difference was found among screen fractions for DAP samples, and reasonably good agreement was found among a ground sample, the 3 screen fractions, and ultrasonic bath disintegration. Values obtained on unground samples all tended to be higher than on the prepared samples. These data are shown in Table 4.

Some individual steps in the CI procedure were selected for further study. The official method specifies use of Whatman No. 5 filter paper to separate the insoluble residue after the sample is extracted with ammonium citrate. A −20 + 200 mesh portion of TSP sample 1 was used for this test. It was analyzed first by the official procedure and then 5 additional times by substituting Whatman filter papers Nos. 1, 4, 7, 40, and 42. The result by the official method was 1.78% P_2O_5, and ranged from 1.72 to 1.83% P_2O_5 for the other papers. These differences are not significant.

Table 2. Major constituents (%) of size fractions of triple superphosphate Sample 1

Element, %	Screen fraction		
	−20 +40[a]	−40 +100	−100
Total P_2O_5	48.16	48.18	48.23
Ca	16.0	16.1	15.7
Mg	0.36	0.37	0.38
Fe	1.29	1.30	1.30
Al	0.81	0.81	0.82

[a] Passed No. 20 screen, retained on No. 40 screen.

The 2 most common pieces of apparatus for continuously agitating the solution and sample during extraction are a water bath with wrist-action shakers and an air bath with end-over-end tumbling action. The TSP used in the previous trial was analyzed 6 times by each method of agitation. Extraction by end-over-end tumbling yielded an average of 1.77% P_2O_5 with a standard deviation (SD) of 0.04. By wrist-action shakers, the average was 1.67% P_2O_5 (SD 0.03). There was a difference between the averages found, but not of a magnitude that warranted further investigation.

To test the many steps in the official method, a ruggedness test was devised according to the Youden design (3). The variables and the effect of the combinations are shown in Table 5. The differences from these results were Da (0.31), Db (0.10), Dc (0.09), Db (0.16), De (0.15), Df (0.02), and Dg (0.03). These results indicate that size of sample caused a significant difference and that temperature of the citrate bath and time of extraction may be significant factors.

A second ruggedness test was designed excluding the 3 factors noted above as contributing to imprecision. The conditions chosen for testing and the results are shown in Table 6. The sum differences from these results were: Da (0.03), Db (0.02), Dc (0.06), Dd (0.03), De (0.04), Df (0.07), and Dg (0.06). These results indicate that under the conditions tested these 8 variables did not contribute to imprecise values.

In some instances throughout the study, water-soluble P_2O_5 was determined on size fractions, unground samples, ultrasonically disintegrated samples, etc.; without exception, the results obtained were in inverse ratio to the CI P_2O_5 found in the sample. This finding was

Table 3. Cl P_2O_5 (%) in ground fractions, unground, and ultrasonic-disintegrated triple superphosphate samples

	Ground						
Sample	−20 +40[a]	−40 +100	−100 +200	−200 +325	−325	Unground[b]	Ultrasonic
1	1.77	1.77	1.89	1.15	0.78	1.74	1.77
2	1.70	1.95	1.83	0.96	0.73	1.75	—
3	1.28	1.50	0.51	0.12	0.07	1.00	0.91
4	1.42	1.75	1.77	0.75	0.43	1.51	1.49
5	0.39	0.44	0.44	0.45	0.48	0.53	—

[a] Passed No. 20 sieve, retained on No. 40 sieve.
[b] Average of 2 independent determinations.

Table 4. Cl P_2O_5 (%) in selected diammonium phosphate samples

	Ground					
Sample	Mixed	−20 +100[a]	−100 +325	−325	Unground	Ultrasonic
1	0.54	0.58	0.50	0.38	0.71	0.55
2	0.27	0.25	0.21	0.19	0.44	0.27
3	0.64	0.76	0.70	0.64	0.80	0.69

[a] Passed No. 20 sieve, retained on No. 100 sieve.

Table 5. Variables and results for ruggedness test No. 1

Key		
Variable	Designation	Values
Sample weight, g	A,a	1.2, 0.8
Citrate pH	B,b	7.1, 6.9
Initial citrate temp., °C	C,c	65, 27
Bath temp., °C	D,d	70, 60
Extraction time, min	E,e	70, 50
Water-sol. wash time, min	F,f	75, 45
Final wash temp., °C	G,g	65, 27

Results		
Condition No.	Combination	Cl P_2O_5, %
1	A,B,C,D,E,F,G	1.86
2	A,B,c,D,e,f,g	2.09
3	A,b,C,d,E,f,g	1.91
4	A,b,c,d,e,F,G	2.16
5	a,B,C,d,e,F,g	1.83
6	a,B,c,d,E,f,G	1.82
7	a,b,C,D,e,f,G	1.61
8	a,b,c,D,E,F,g	1.51
Av.		1.85
Std dev.		0.22
Range		0.65

Table 6. Variables and results for ruggedness test No. 2

Key		
Variable	Designation	Values
Water-sol. wash time, min	A,a	75, 45
Citrate pH	B,b	7.1, 6.9
Citrate sp. gr.	C,c	1.095, 1.085
Temp. citrate measured, °C	D,d	65, 27
Filter paper	E,e	No. 2 pulp, No. 42 paper
Final water wash, mL	F,f	300, 200
Final wash temp. °C	G,g	65, 27

Results		
Condition No.	Combination	Cl P_2O_5, %
1	A,B,C,D,E,F,G	1.71
2	A,B,c,D,e,f,g	1.73
3	A,b,C,d,E,f,g	1.66
4	A,b,c,d,e,F,G	1.72
5	a,B,C,d,e,F,g	1.57
6	a,B,c,d,E,f,G	1.83
7	a,b,C,D,e,f,G	1.64
8	a,b,C,D,E,F,g	1.74
Av.		1.70
Std dev.		0.077
Range		0.26

consistent with the results previously reported (4).

Conclusions and Recommendations

The official method for the determination of citrate-insoluble phosphorus in triple superphosphate is greatly affected by the fineness of the ground sample. Therefore, it is mandatory that samples be extremely well homogenized after grinding.

Other factors that may affect reproducible results for CI P_2O_5 are size of sample, temperature of citrate bath, and extraction time.

Factors that had previously been thought to be highly critical in the CI P_2O_5 method were found to be reasonably rugged. These included pH and specific gravity of the citrate, filter medium used to separate citrate-insoluble residue, and temperature of the final wash water.

References

(1) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **2.044-2.046**

(2) Dillard, E. F., Frazier, A. W., Woodis, T. C., Jr, & Achorn, F. P. (1981) *Bulletin Y-162*, Tennessee Valley Authority, National Fertilizer Development Center, Muscle Shoals, AL

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

(4) Johnson, F. J., & Farley, J. A. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 702-705

MYCOTOXINS

Susceptibility of Strawberries, Blackberries, and Cherries to *Aspergillus* Mold Growth and Aflatoxin Production

GERALD C. LLEWELLYN, THOMAS EADIE,[1] and WILLIAM V. DASHEK[2]
Virginia Commonwealth University, Department of Biology, Richmond, VA 23284

The susceptibility of blackberries, cherries, and strawberries to *Aspergillus* growth and aflatoxin production has been examined. Three aflatoxigenic isolates of *Aspergillus*, *A. flavus* ATCC 15548 and NRRL 3251 as well as *A. parasiticus* NRRL 2999, were cultured on homogenates of the fruits for 14 days at 28 ± 2°C. Percent mycelial growth and spore infestation were determined each day with a calibrated grid. At day 14 each culture was frozen at −5°C until aflatoxins were extracted with methylene chloride and water. Aflatoxins were separated by thin layer chromatography (TLC) with benzene–methanol–acetic acid (90 + 5 + 5). This extraction and solvent system provided satisfactory separations of the aflatoxins and was free of background interference on the TLC plates. Although all fruits served as substrates for both *Aspergillus* growth and aflatoxin production, cherries appeared to be a more favorable substrate than did blackberries, and the latter was more favorable than strawberries. Whereas *A. flavus* produced both B_1 and G_1 on all substrates, it yielded B_2 and G_2 only on cherries. Although *A. parasiticus* NRRL 2999 synthesized B_1, B_2, G_1, and G_2 on both blackberries and cherries, no aflatoxins were detected on strawberries. In contrast, *A. flavus* NRRL 3251 failed to produce detectable levels of aflatoxin on any substrate. All substrates supported both mycelial growth and subsequent sporulation with cherries > blackberries > strawberries.

Aflatoxins are secondary metabolites of *Aspergillus flavus* and *A. parasiticus* (1, 2). The metabolites are a quadruple threat in that they are hepatocarcinogens, mutagens, teratogens, and toxins. A variety of substrates have been tested for potential growth of aflatoxigenic isolates of *Aspergillus*, including caraway, celery seed, cinnamon, cloves, ginger, mustard, rosemary, and sesame (3). Only a few investigations have been carried out using fruits as substrates. These include intact citrus fruits (4, 5) and dried figs, apricots, pineapples, and raisins (6).

Blackberries (*Eubatus* spp.), cherries (*Prunus* spp.), and strawberries (*Fragaria* spp.) are fruits which result from pollination and subsequent fertilization. Whereas the strawberry is an example of an accessory fruit with achenes on the surface, the blackberry and the cherry are examples of multiple and single drupes, respectively.

These fruits are sold in markets and are also available for the consumer to pick in the field. Because storage and shelf lives of these commodities are quite limited, aged and/or damaged fruit which becomes moldy is removed before selling the non-moldy portion. However, we have observed extensive growth and sporulation of an *Aspergillus*-like mold on cherries displayed at a local supermarket. This observation prompted the present investigation which is concerned with the aflatoxigenic potential of blackberries, cherries, and strawberries.

Experimental

Preparation of Fruit Substrates

Raw, unwashed fruits were used. Strawberries and blackberries were picked locally. Cherries, purchased from a local supermarket, were a Bing Cultivar from Montana. Strawberries and blackberries were not washed and thus contained the natural flora associated with their growing environment. We believe that the cherries were spray-washed by the packer. Five gram lots of the fruits were chopped with a sterile scalpel. Cherries were pitted aseptically before chopping. Chopped fruits with their respective juices were homogenized to a slurry with a sterile mortar and pestle.

Preparation of Aspergillus Isolates

Aspergillus flavus isolate ATCC 15548, *A. flavus* isolate NRRL 3251, and *A. parasiticus* isolate NRRL 2999 were maintained as stock cultures on potato–dextrose–yeast–agar slants as previously described (7). These isolates are known to produce aflatoxins (8).

[1] Commonwealth of Virginia, Department of General Services, Division of Consolidated Laboratory Services, 1 N 14th St, Richmond, VA 23219.

[2] West Virginia University, Department of Biology, Morgantown, WV 26506.

Received September 9, 1981. Accepted December 8, 1981.
This paper was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

0004-5756/82/6503-0659–06$01.00

Inoculation of Fruit Substrates

Five gram wet-weight batches of each substrate were placed in 60 mL sterile Pyrex prescription bottles with both flat and rounded sides. Bottles containing the substrates were then transferred to a negative pressure hood which had been sterilized with both 25% sodium hypochlorite and a 30-min exposure to ultraviolet light. Substrates were inoculated with equal aliquots of spores taken from single slants, and inoculated substrates were incubated in the dark for 14 days at 25 ± 2°C.

Growth Rate Quantification

To determine percentage mycelial growth and spore infestation, a clear plastic grid consisting of 100 squares spaced over a 60 × 35 mm area was placed over each prescription bottle. Completely filled squares viewed below but through the grid were counted as 1% each. Except for day 4, determinations were carried out daily for 2 weeks.

Replication of Experiment

Data within the tables represent means and standard deviations of triplicate observations. All experiments were performed in triplicate, and triplicate determinations were made within each experiment.

Aflatoxin Analysis

At day 14, each culture was observed, capped, and frozen at −5°C until tested. Contents of individual culture bottles including substrate, mycelia, and spores were extracted and analyzed. Methylene chloride (25 mL) and water (2 mL) were added to the frozen contents of each bottle, which was then allowed to come to room temperature. Each bottle was capped following addition of the extracting solvents. All bottles were shaken vigorously on a mechanical wrist-action shaker for 1 h followed by gravity filtration through Whatman No. 2V paper. Aliquots (10 mL) of each filtrate were evaporated under nitrogen for thin layer chromatography (TLC) on Adsorbosil-Plus 1, hard layer plates (Applied Science Laboratories, State College, PA 16801), according to AOAC methods (9). The alternative developing solvent, benzene–methanol–acetic acid (90 + 5 + 5), yielded excellent separations of the aflatoxins and was free of background interference. Accordingly, this solvent was used instead of the specified acetone–$CHCl_3$ (1 + 9) developing solvent. Data are reported as ng/g substrate. Separate control samples of each fruit substrate used in the study were tested before experimental use to confirm the absence of aflatoxins or interfering materials. Visual readings were made for each determination by comparing fluorescent spots with reference samples and making the necessary dilutions of the spotted extracts until they matched.

Results

Aflatoxin Synthesis by Aspergillus Isolates on Fruits

Blackberries (*Eubatus*).—Markedly divergent results were observed on inoculation and subsequent incubation of blackberry homogenates with 3 *Aspergillus* isolates (Table 1). Whereas nearly equivalent amounts of B_1 and G_1 were formed by *A. flavus* isolate ATCC 15548, no aflatoxins were synthesized by *A. flavus* isolate NRRL 3251 when blackberry was used as a substrate. Placement into and maintenance of blackberry homogenates with *A. parasiticus* isolate NRRL 2999 yielded approximately 3 times as much B_1 as G_1.

Strawberries (*Fragaria*).—When strawberry homogenates were inoculated and incubated with spores of *A. flavus* isolate ATCC 15548, both B_1 and G_1 were synthesized, with slightly more of the latter (57% of the total toxin recovered) than the former (43% of the total toxin recovered) formed (Table 1). In contrast, neither *A. parasiticus* isolate NRRL 2999 nor *A. flavus* isolate NRRL 3251 produced any aflatoxin on strawberry homogenates.

Cherries (*Prunus*).—Although inoculation and then incubation of cherry homogenates with *A. flavus* isolate NRRL 3251 did not result in any aflatoxin, procedures using *A. flavus* isolate ATCC 15548 resulted in nearly equivalent amounts of B_1 and G_1 (Table 1). In contrast, 98 times more B_1 than G_1 was synthesized when cherry homogenates were subjected to and then cultured with *A. parasiticus* isolate NRRL 2999.

The results presented in Table 1 revealed that of the 3 fruits, strawberries appear to be the least favorable substrate for aflatoxin production; cherries seem to be the most favorable, with blackberries intermediate between the two.

Spore Infestation on Fruit Substrates

Blackberries.—Dissimilar results for percentage substrate area covered by spores were observed when blackberry homogenates were inoculated with 3 aflatoxigenic *Aspergillus* isolates (Table 2). Whereas only 15% of the homogenate was covered by spores 5 days after inoculation and incubation with *A. flavus* isolate ATCC 15548,

Table 1. Aflatoxin synthesis by 3 *Aspergillus* isolates on 3 fruits [a]

Aflatoxin	*A. flavus* ATCC 15548, ng/g	% of total	*A. parasiticus* NRRL 2999, ng/g	% of total	*A. flavus* NRRL 3251, ng/g	% of total
			Blackberries			
B_1	7551 ± 5041	46	3175 ± 2848	71	ND[b]	—
B_2	ND	—	30 ± 52	<1	ND	—
G_1	8842 ± 6842	54	1237 ± 1195	28	ND	—
G_2	ND	—	12 ± 21	<1	ND	—
Total	16 393		4454		—	
			Strawberries			
B_1	111 ± 46	43	ND	—	ND	—
B_2	ND	—	ND	—	ND	—
G_1	149 ± 50	57	ND	—	ND	—
G_2	ND	—	ND	—	ND	—
Total	260		—		—	
			Cherries			
B_1	21 345 ± 1667	48	154 862 ± 15 266	98	ND	—
B_2	347 ± 65	<1	1924 ± 544	1	ND	—
G_1	22 711 ± 463	51	122 ± 14	<1	ND	—
G_2	468 ± 73	<1	1114 ± 601	<1	ND	—
Total	44 871		158 022		—	

[a] Means and standard deviations from $CHCl_3$-extracted fruit substrates for 3 replicate experiments with 3 replicates within each experiment.
[b] ND = none detected.

percentages of 70 and 80 were seen at 2 and 3 days with *A. parasiticus* isolate NRRL 2999 and *A. flavus* isolate NRRL 3251, respectively. Between the 6th and 7th days, the substrate area covered by spores rose from 50 to 80% and then to 100% between days 8 and 9 for homogenates inoculated and incubated with *A. flavus* isolate ATCC 15548. In contrast, isolates NRRL 2999 and NRRL 3251 yielded changes of 90 to 100% and 95 to 100% between days 3 and 5 and between 8 and 9, respectively.

Strawberries.—Whereas 50% of the strawberry homogenate was covered with spores 3 days post inoculation and incubation with *A. flavus* isolate ATCC 15548, 60% was covered at 2 days with *A. parasiticus* isolate NRRL 2999 and *A. flavus* isolate NRRL 3251. The substrate area covered by spores increased from 60 to 70% (between days 2 and 3) and 75 to 80% (between days 7 and 8) for the former isolate and 60 to 80% (between days 2 and 3) and 80 to 85% (between days 5 and 6) for the latter isolate.

Cherries.—Although the percentage of substrate area covered by spores did not increase until the 6th day (75%) after inoculation and incubation of cherry homogenates with *Aspergillus* isolate ATCC 15548, these percentages increased to 80 and 90 by day 3 with *Aspergillus* isolates *parasiticus* NRRL 2999 and *flavus* NRRL 3251, respectively. A 100% substrate area covered by spores was reached on days 14 (isolate ATCC 15548), 7 (isolate NRRL 2999), and 5 (isolate NRRL 3251).

Mycelial Growth on Fruit Substrates

Blackberries.—The area of blackberry homogenate covered by mycelia was ≥70% when the homogenates were inoculated and incubated for 2 days with *Aspergillus* isolate ATCC 15548, NRRL 2999, or NRRL 3251 (Table 3). Between days 2 and 3 the area of substrate occupied by mycelia increased from 70 to 90% and from 80 to 85% and then remained constant for the next 11 days when blackberry homogenates were treated with spores of either *A. flavus* isolate ATCC 15548 or *A. parasiticus* isolate NRRL 2999, respectively, and subsequently maintained. In contrast, for inoculation and incubation with *A. flavus* NRRL 3251, area covered by mycelia increased from 85 to 90, 90 to 95, and 95 to 100% between days 2 and 3, 5 and 6, and 7 and 8, respectively.

Strawberries.—When *Aspergillus* isolate ATCC 15548, NRRL 2999, or NRRL 3251 was introduced into and cultured on strawberry homogenates for 2 days, ≥50% of the homogenates were covered with mycelia. Between days 2 and 3, the area

Table 2. Mycelial substrate area (%) covered by spores

Fruit	*Aspergillus* isolate	Days after inoculation													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Blackberry	*A. flavus* ATCC 15548	—[a]	0	0	—[a]	15	50	80	80	100	100	100	100	100	100
	A. parasiticus NRRL 2999	—	70	90	—	100	100	100	100	100	100	100	100	100	100
	A. flavus NRRL 3251	—	—	80	—	90	95	95	95	100	100	100	100	100	100
Strawberry	*A. flavus* ATCC 15548	—	0	50	—	50	50	50	50	50	50	50	75	75	75
	A. parasiticus NRRL 2999	—	60	75	—	75	75	75	80	80	80	80	80	80	100
	A. flavus NRRL 3251	—	60	80	—	80	85	85	85	85	85	85	85	85	85
Cherry	*A. flavus* ATCC 15548	—	0	0	—	0	75	75	75	75	75	80	80	80	100
	A. parasiticus NRRL 2999	—	0	80	—	80	95	100	100	100	100	100	100	100	100
	A. flavus NRRL 3251	—	0	90	—	100	100	100	100	100	100	100	100	100	100

[a] No observations made.

Table 3. Substrate area (%) covered by mycelia

Fruit	*Aspergillus* isolate	Days after inoculation													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Blackberry	*A. flavus* ATCC 15548	—[a]	70	90	—[a]	90	90	90	90	90	90	90	90	90	90
	A. parasiticus NRRL 2999	—	80	85	—	85	85	85	85	85	85	85	85	85	85
	A. flavus NRRL 3251	—	85	90	—	90	95	95	100	100	100	100	100	100	100
Strawberry	*A. flavus* ATCC 15548	—	50	75	—	80	80	80	80	80	80	80	80	80	80
	A. parasiticus NRRL 2999	—	50	75	—	75	75	75	80	80	80	80	80	80	80
	A. flavus NRRL 3251	—	60	80	—	80	80	80	80	80	80	80	80	80	80
Cherry	*A. flavus* ATCC 15548	—	95	95	—	100	100	100	100	100	100	100	100	100	100
	A. parasiticus NRRL 2999	—	95	95	—	95	100	100	100	100	100	100	100	100	100
	A. flavus NRRL 3251	—	80	90	—	90	90	95	100	100	100	100	100	100	100

[a] No observation made.

Table 4. Summary of time-dependent changes in spore infestation and mycelial growth related to aflatoxin synthesis by 3 aflatoxigenic *Aspergillus* isolates grown on blackberry, strawberry, and cherry substrates

Fruit	*A. flavus* ATCC 15548	*A. parasiticus* NRRL 2999	*A. flavus* NRRL 3251
	Aflatoxin Type Synthesized, % of Total		
Blackberry	46 B_1 54 G_1	71 B_1 28 G_1	ND B_1 ND G_1
Strawberry	43 B_1 57 G_1	ND B_1 ND G_1	ND B_1 ND G_1
Cherry	48 B_1 51 G_1	98 B_1 1 G_1	ND B_1 ND G_1
	Mycelial Growth, %		
Blackberry			
%	0–70 70–90	0–80, 80–85	0–85 85–100
Days	0–2 2–14	0–2 2–14	0–2 2–14
Strawberry			
%	0–50 50–80	0–50 50–80	0–60 60–80
Days	0–2 2–14	0–2 2–14	0–2 2–14
Cherry			
%	0–95 95–100	0–95 95–100	0–80 80–100
Days	0–2 2–14	0–2 2–14	0–2 2–14
	Spore Infestation		
Blackberry			
%	0–15 15–100	0–70 70–100	0–80 80–100
Days	0–5 5–14	0–2 2–14	0–3 3–14
Strawberry			
%	0–50 50–75	0–60 60–100	0–60 60–85
Days	0–3 3–14	0–2 2–14	0–2 2–14
Cherry			
%	0–75 75–100	0–80 80–100	0–90 90–100
Days	0–6 6–14	0–3 3–14	0–3 3–14

covered by mycelia accelerated from 50 to 75% for *A. flavus* ATCC 15548 and *A. parasiticus* NRRL 2999, but for *A. flavus* NRRL 3251 the enhancement was from 60 to 80%. Whereas the substrate area covered by mycelia did not increase during 14 days for strawberry homogenates inoculated and incubated with *A. flavus* NRRL 3251, the area did increase from 75 to 80% in those homogenates subjected to and cultured with either *A. flavus* isolate ATCC 15548 or *A. parasiticus* isolate NRRL 2999. The promotions were between days 3 and 5 for the former and days 7 and 8 for the latter.

Cherries.—The area of cherry homogenate covered by mycelia was ≥80% following inoculation and incubation for 2 days with *Aspergillus* isolate ATCC 15548, NRRL 2999, or NRRL 3251. The area increased from 95 to 100% between days 3 and 5, 5 and 6, and 7 and 8 when homogenates were inoculated and incubated with spores of isolates *A. flavus* ATCC 15548, *A. parasiticus* NRRL 2999, and *A. flavus* NRRL 3251, respectively.

Correlations Between Aflatoxin Synthesis and Area Covered by Mycelia and Spores

Table 4 summarizes the time-dependent changes in mycelial growth and spore infestation as related to aflatoxin synthesis by the 3 aflatoxigenic *Aspergillus* isolates. There does not appear to be a correlation between total quantity of aflatoxins, type of aflatoxins, and either percentage of mycelial growth or sporulation.

Discussion

Table 5 compares the aflatoxigenic rankings for fruits and *Aspergillus flavus* ATCC 15548 as well as *A. parasiticus* NRRL 2999. It is apparent that the substrates differ with respect to the type of aflatoxin produced by *A. flavus* and *A. parasiticus*. For *A. flavus* the rankings for B_1 were cherry > blackberry > strawberry. In contrast, B_2 was produced only on cherry and blackberry with the former serving as a more effective substrate than the latter. The rankings for G_1 were strawberry > blackberry > cherry. Aflatoxin G_2 yields were observed only on cherry. The rankings for *A. parasiticus* were B_1 and B_2, cherry > blackberry; G_1, blackberry > cherry; and G_2, cherry and blackberry about the same.

A. flavus isolate 3251 was a consistent nonproducer of aflatoxin in this study although it both grew and sporulated well. These results agree with those obtained when the isolate was grown on spices (3).

Table 5. Aflatoxigenic rankings for fruits and *Aspergillus parasiticus* NRRL 2999 and *A. flavus* ATCC 15548 [a]

Rank	Aflatoxin production			
	B_1	B_2	G_1	G_2
	A. flavus ATCC 15548			
1	cherry	cherry	strawberry	cherry
2	blackberry	blackberry	blackberry	
3	strawberry		cherry	
	A. parasiticus NRRL 2999			
1	cherry	cherry	blackberry	cherry–blackberry
2	blackberry	blackberry	cherry	

[a] Rankings determined from data in Table 1.

A number of factors which have not been considered in the present investigation may influence mycelial growth, sporulation, and aflatoxin production. These factors are necessary nutrients from the substrate, competing microorganisms in the substrate, inhibitory chemicals, natural barriers such as seed coat, water content, pH, temperature, and length of time for the mold to grow (3).

In summary, the fruits studied, especially cherries and blackberries, are susceptible to toxigenic mold growth. Routine but slightly abbreviated methods for aflatoxin analysis appear satisfactory. We do not recommend monitoring such fruits for aflatoxins when the former are selected by the individual consumer, but if mass harvesting and processing are involved, then an occasional analysis of blackberries and cherries appears to be warranted. Strawberries do not appear to be an aflatoxigenic supportive substrate. They may even be inhibitory to toxin production but are not antimycotic.

Acknowledgments

We thank Roland Guthrie for providing botanical expertise, and Michael Amos for some data analysis and editorial assistance. Nancy Bew contributed extensively in the experimental and technical data collection aspects of this study. We thank A. David Lynn and Deborah C. Paul, Food and Animal Remedies Section, Bureau of Product Regulation, Division of Consolidated Laboratory Services, for their cooperation.

References

(1) Butler, W. H. (1974) in *Mycotoxins*, I. F. H. Purchase (Ed.), Elsevier Scientific Publishing Co., Amsterdam, The Netherlands, pp. 1–28

(2) Fewell, A. J. (1969) in *Aflatoxin—Its Scientific Background, Control and Implication*, L. A. Goldblatt (Ed.), Academic Press, New York, NY, pp. 187–222

(3) Llewellyn, G. C., Burkett, M. L., & Eadie, T. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 955–960

(4) Alderman, G. C., & Marth, E. H. (1974) *J. Milk Food Technol.* **37,** 308–313

(5) Alderman, G. C., & Marth, E. H. (1974) *J. Milk Food Technol.* **37,** 451–456

(6) Morton, S. G., Eadie, T., & Llewellyn, G. C. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 958–962

(7) Llewellyn, G. C., Carlton, W. W., Robbers, J. E., & Hansen, W. G. (1973) *Dev. Ind. Microbiol* **14,** 325–330

(8) Hilldrup, J. L., Eadie, T., & Llewellyn, G. C. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 96–99

(9) *Mycotoxins Methodology* (1980) AOAC, Arlington, VA, secs **26.013** and **26.031**

High Pressure Liquid Chromatographic Determination of Aflatoxins by Using Radial Compression Separation

ERIC C. SHEPHERD, TIMOTHY D. PHILLIPS,[1] NORMAN D. HEIDELBAUGH and A. WALLACE HAYES

Texas A&M University, Department of Veterinary Public Health, College Station, TX 77843

A rapid method for the determination of aflatoxins was developed using high pressure liquid chromatography and a radial compression separation system. A standard solution of aflatoxins B_1, B_2, G_1, G_2, and M_1 was analyzed at flow rates of 2.0 and 6.0 mL/min. Retention times, peak heights, and peak areas were reproducible over a 3-day period. Coefficients of variation for aflatoxin B_1 at 2.0 and 6.0 mL/min were, respectively, 1.04 and 0.87% (retention time); 2.9 and 4.7% (peak height); and 8.2 and 4.7% (peak area). At 6.0 mL/min there was an approximate 25% loss in sensitivity but a greater than 50% reduction in retention time. Separation of all the aflatoxins was excellent using a dual flow rate of 2.0 mL/min with a change to 8.0 mL/min at 15 min post-injection. The applicability of the radial compression separation system for the rapid determination of aflatoxins in human tissues was also tested. Spiked samples of liver, serum, and urine showed good resolution of all aflatoxin peaks at the higher flow rates.

The aflatoxins are a group of toxic fungal metabolites produced by several species of *Aspergillus*. There are at least 16 naturally occurring aflatoxins, but the 4 major toxins are aflatoxins B_1, B_2, G_1, and G_2 (1). Aflatoxin B_1 is a potent hepatocarcinogen (2) and mutagen (3) that has been found in a variety of foods and feeds (4). Its consumption has been linked to human diseases such as primary hepatocellular carcinoma and Reyes syndrome (5). The public health importance and widespread occurrence of aflatoxin B_1 has stimulated the development of numerous chromatographic methods of analysis.

Early methods for aflatoxin analysis involved extraction into intermediate polarity solvents, separation by thin layer chromatography (TLC), and visualization of the characteristic fluorescent spot(s) (6). However, although suited to mass screening, TLC lacks the precision and sensitivity of high pressure liquid chromatography (HPLC). Many HPLC methods have been developed for determining aflatoxins. Normal phase methods, such as the one developed by Pons (7), have the advantages of wide applicability, good resolution of the majority of the aflatoxins, and detection in the range 1–2 ng/g. Reverse phase methods using fluorescence detection of the aflatoxins have also been developed (8–10). These methods have the advantages of increased sensitivity, solvent safety, and increased column stability. The major limitation of fluorescence detection is the possibility of errors due to quenching from contaminant compounds.

The wide range in polarity between the 4 major aflatoxins and their metabolites has made it difficult to develop an ideal HPLC mobile phase solvent system that will give optimum resolution. The closest to an ideal solvent system for normal phase HPLC separation of the aflatoxins has been described by Pons (7).

In this study, we report an adaptation of the Pons HPLC method using radial compression separation with UV detection at 365 nm, and its application in the determination of aflatoxins from human tissues and biological fluids.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Waters Model ALC-204 HPLC system (Waters Associates, Inc., Milford, MA) equipped with M-6000 and M-45 pumps, U6K septumless injector, Model 440 UV absorbance detector with 365 nm primary filter in series with Model 420-E fluorescence detector (365 nm excitation, 425 nm emission), Model 970-A variable wavelength UV–visible detector (Tracor Instruments, Austin, TX), and Model 660 solvent programmer (Waters).

(b) *HPLC column.*—Waters radial compression separation system consisting of RCM-100 radial compression module and normal phase Radial-Pak cartridge (10 μm particle size).

(c) *Tissue homogenizer.*—Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY).

(d) *Liquid scintillation spectrophotometer.*—Beckman LS 7500 (Beckman Instruments, Palo Alto, CA).

(e) *HPLC recorder.*—Houston Instrument Series B-5000 dual-pen Omni-Scribe (Austin, TX).

[1] Address correspondence to this author.

Received September 1, 1981 Accepted November 2, 1981.

This study was supported by Texas Agricultural Experiment Station Projects H6215, AH6397, and AH6529

0004-5756/82/6503-0665-07$01.00

(f) *Integrator.*—Hewlett-Packard Model 3390 A (Avondale, PA).

Reagents

(a) *Aflatoxins.*—Aflatoxins B_1, B_2, G_1, G_2, and M_1 (Sigma Chemical Co., St. Louis, MO). Purity was confirmed by TLC and HPLC. ^{3}H-Aflatoxin B_1 (15 Ci/mmol) was obtained from Moravek Biochemicals (City of Industry, CA).

(b) *Solvents.*—$CHCl_3$, acetone, cyclohexane, acetonitrile, 2-propanol, and hexane (Burdick & Jackson, Muskegon, MI); triple distilled and demineralized water.

(c) *HPLC mobile phase.*—Water-saturated $CHCl_3$–cyclohexane–acetonitrile (75 + 22.5 + 2.5) with 2% 2-propanol.

(d) *Scintillation fluid.*—Beckman Ready-Solv HP (Beckman Instruments).

Tissues and Biological Fluids

Human liver tissues (10–20 g each) were obtained at autopsy through the cooperation of Ralph M. Garruto, National Institutes of Health, Bethesda, MD. Human plasma and urine samples were collected from laboratory staff.

Extraction of Samples

Samples of human liver, plasma, and urine were spiked with aflatoxins B_1, B_2, G_1, G_2, and M_1 at levels of 32, 38.4, 44.8, 38.4, and 320 ng/g, respectively. Spiked samples and unspiked controls were extracted according to the method of Siraj and coworkers (11) as follows: Briefly, weigh 2.5 g samples in 50 mL screw-top tubes. Add 8 mL water to liver, plasma, or urine samples and mix. Homogenize liver sample 20–30 s with Polytron tissue homogenizer. To all tubes add 30 mL acetone, mix well, and centrifuge 10 min at 1500 rpm. Transfer supernatant fluid to clean 50 mL tube. To liver sample add 2 mL saturated lead acetate solution. Centrifuge tubes and transfer supernatant fluid to clean test tube. To all samples add 10 mL hexane to remove residual lipids. Extract aflatoxins from the aqueous acetone with 3 successive 10 mL aliquots of $CHCl_3$. Combine $CHCl_3$ aliquots and add 5 mL 5% NaCl solution. Mix well, centrifuge, and then discard the NaCl solution. Evaporate $CHCl_3$ extracts to dryness under nitrogen. Redissolve in 100 μL Pons solution (HPLC mobile phase) and inject 20 μL onto the HPLC column.

Recovery and loss of aflatoxin B_1 at key steps in the extraction procedure were checked by using tritium-labeled aflatoxin B_1. Liver samples (2–3 g) were spiked with 14.2 μCi ^{3}H-aflatoxin B_1, and then extracted. Extracted samples and labeled standard were separated by HPLC and the effluent was collected at 12 s intervals into 7 mL scintillation vials. Vials were dried under gentle stream of nitrogen, and then 4 mL scintillant was added and the contents of the vials were mixed. Counts per minute (cpm) were converted to disintegrations per minute (dpm), using automatic internal standardization and a previously prepared quench correction curve.

HPLC Procedure

Standards and extracted tissues were analyzed by HPLC using the conditions established by Pons (6, 7) for normal phase chromatography of the aflatoxins. This system specifies a normal phase silica gel column and a mobile phase of $CHCl_3$–cyclohexane–acetonitrile and 2-propanol.

Pure aflatoxin standards were run at flow rates of 2 and 6 mL/min. Comparisons were made of retention times, peak heights, and peak areas with standard solutions. Linearity of response and minimum detection level were determined by injecting standard solutions at levels of 0.5–60 ng.

Extracts of control and spiked tissues were analyzed at a flow rate of 6 mL/min. Aflatoxin standards, control liver, and spiked liver samples were also analyzed by a dual flow rate program.

Results and Discussion

Effect of Flow Rate Changes

The effect of flow rate on column pressure, retention time, and capacity ratio for the resolution of aflatoxins B_1, B_2, G_1, G_2, and M_1 is shown in Table 1. Column pressure ranged from 50 psi at a flow rate of 1 mL/min to a maximum of 2600 psi at a flow rate of 9.9 mL/min with the radial compression module. This system is more versatile than conventional HPLC columns where flow rates of 1–2 mL/min produce pressures in the range of 1000–2000 psi. The low pressures exhibited with the radial compression module permit changes in flow rate to be used as a determinant in shortening aflatoxin analysis time.

At a flow rate of 1 mL/min there was good separation of the aflatoxin peaks, but the extreme peak broadening and long analysis time make this flow rate impractical. At flow rates of 2 and 4 mL/min the resolution of the aflatoxins was good, but the long retention time for M_1 still makes the analysis of this component tedious. At flow rates above 6 mL/min the retention time

Table 1. Effect of increasing flow rate on column pressure, retention time, and capacity ratio for resolution of aflatoxin [a] by HPLC with radial compression module

Flow rate, mL/min	Pressure, psi	Retention time, min					Capacity ratio, k' [b]				
		B_1	B_2	G_1	G_2	M_1	B_1	B_2	G_1	G_2	M_1
1.0	50	15.8	19.6	24.9	31.5	—	5.3	6.8	8.9	11.6	—
2.0	210	6.0	7.2	9.2	11.3	55.3	3.1	3.8	5.2	6.6	36.5
4.0	600	3.1	3.7	4.8	5.8	27.7	2.9	3.7	5.1	6.4	34.5
6.0	1000	2.2	2.6	3.3	4.0	19.6	3.5	4.3	5.7	7.1	39.0
8.0	1400	1.7	2.1	2.5	3.1	14.7	4.8	6.2	7.6	9.6	49.6
9.9	2600	1.3	1.6	2.1	2.4	11.3	5.5	7.0	9.5	11.0	55.5

[a] 20 μL of a standard aflatoxin mixture of B_1 (20 ng), B_2 (24 ng), G_1 (28 ng), G_2 (24 ng), and M_1 (200 ng) dissolved in Pons solution.

[b] $k' = V_1 - V_0/V_0$ where V_1 = peak elution volume and V_0 = void volume.

for aflatoxin M_1 is short enough to be useful for rapid analysis, but the resolution of aflatoxins B_1, B_2, G_1, and G_2 deteriorates. The capacity ratio, k', is indicative of the relative retention and resolution of a compound. A k' value in the range of 2–6 tends to optimize resolution and is usually changed by varying the solvent strength. The data in Table 1 show that acceptable capacity ratios were obtained for aflatoxin B_1 at all flow rates tested. Representative chromatograms at flow rates of 2, 4, and 6 mL/min are shown in Figure 1.

Reproducibility of Peak Parameters

Retention time reproducibility for aflatoxins B_1 and G_1 at flow rates of 2 and 6 mL/min is shown in Table 2. Reproducibility was good at both flow rates as indicated by the coefficients of variation of 1.04 and 1.08% at 2 mL/min and 0.87 and 1.12% at 6 mL/min.

Precision was evaluated at both flow rates by injecting ten 20 μL aliquots of a mixed aflatoxin standard containing aflatoxins B_1 (20 ng) and G_1 (28 ng). The calculated results for these injections are shown in Table 3. Reproducibility of peak height and peak area at both flow rates (2 and 6 mL/min) was acceptable. There was a 25–30% decrease in peak height and peak area values when the flow rate was increased from 2 to 6 mL/min. To determine the detection limit at 2 and 6 mL/min, a standard solution of aflatoxin B_1 was injected at levels from 0.5 to 60 ng.

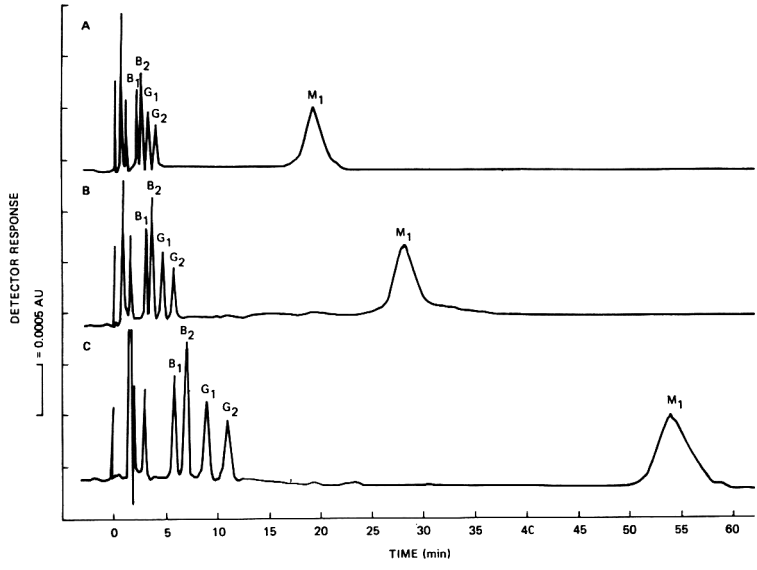


Figure 1. Representative liquid chromatograms of mixed aflatoxin standard (B_1, 20 ng; B_2, 24 ng; G_1, 28 ng; G_2, 24 ng; M_1, 200 ng) separated at 3 flow rates (A, 6 mL/min; B, 4 mL/min; C, 2 mL/min). Separation on 10 μm silica gel with Pons solution as mobile phase and detection at 365 nm, sensitivity at 0.005 AUFS.

Table 2. Reproducibility of retention times in HPLC determination of aflatoxins B_1 and G_1 at 2 flow rates[a]

	2 mL/min		6 mL/min	
Statistic	B_1	G_1	B_1	G_1
Injections, *N*	10	10	10	10
Retention time (s)				
Range	336–488	496–513	128–132	197–204
Mean	343.6	509.0	129.5	200.9
SD	3.6	5.5	1.13	2.26
CV (%)[b]	1.04	1.08	0.87	1.12
Mean retention time (min)	5.7	8.5	2.1	3.3

[a] Repeated 20 μL injections of a standard aflatoxin mixture containing 20 ng B_1 and 28 ng G_1 assayed over a 3-day period.
[b] Coefficient of variation (%) = std dev./mean × 100.

There was a linear detector response at both flow rates with correlation coefficients of 0.996 and 0.997, respectively. The calibration curves and the data in Table 3 indicated that aflatoxin B_1 could be detected at levels less than 1 ng at a 2 mL/min flow rate and 1.5 ng at 6 mL/min. The minimum amounts that could be accurately quantitated were 1.6 ng at 2 mL/min and 2.2 ng at 6 mL/min.

Determination of Aflatoxins in Human Urine, Serum, and Liver

The applicability of radial compression separation in the rapid analysis of aflatoxins in human tissues was tested at a flow rate of 6 mL/min. Samples of human urine, serum, and liver were analyzed by HPLC with and without the addition of a mixed aflatoxin standard containing B_1, B_2, G_1, G_2, and M_1. HPLC analysis of the ^{3}H-aflatoxin B_1 used to spike liver tissue showed a single peak of radioactivity corresponding to a single absorption peak at 365 nm (Figure 2). Recovery of ^{3}H-aflatoxin B_1 from 5 individually spiked liver samples averaged 84.4%. Analysis of intermediate steps in the extraction procedure showed that 9% of the radioactivity was lost in the hexane wash and the final aqueous solution left after $CHCl_3$ partition.

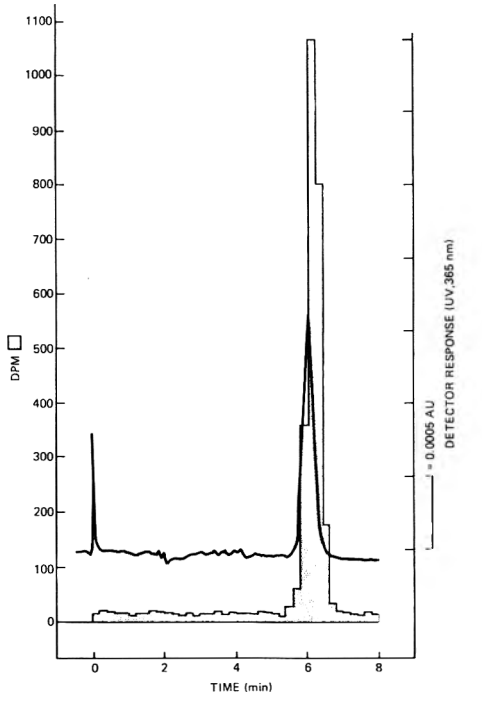


Figure 2. Liquid chromatogram of aflatoxin B_1 standard (25 ng) containing 2 nCi ^{3}H-aflatoxin B_1 (15 Ci/mMole). Column effluent analyzed at 365 nm and then collected at 12 s intervals for measurement of radioactivity. Separation on 10 μm silica gel with Pons solution at a flow rate of 2 mL/min.

Table 3. Reproducibility of peak height and peak area in HPLC determination of aflatoxins B_1 and G_1 at 2 flow rates[a,b]

	2 mL/min		6 mL/min	
Statistic	B_1	G_1	B_1	G_1
Injections, *N*	10	10	10	10
Peak height (mm)				
Range	85–95	66–73	63–73	45–50
Mean	90.3	68.5	67.2	48.7
SD	2.7	3.0	3.2	2.4
CV (%)	2.9	4.3	4.7	5.0
Sensitivity (mm/ng)[c]	4.5	2.4	3.4	1.7
Peak area (mm^2)				
Range	128–164	157–187	94–109	135–161
Mean	142	172	101	146
SD	11.7	10.2	4.8	7.14
CV (%)	8.2	5.9	4.7	4.8
Sensitivity (mm^2/ng)[c]	7.1	6.1	5.04	5.21

[a] Repeated 20 μL injections of a standard aflatoxin mixture; refer to Table 2.
[b] Chart speed = 0.2 in./min.
[c] Calculated at maximum sensitivity, 0.005 AUFS.

Representative liquid chromatograms of extracted samples are presented in Figures 3–5. Figure 3 shows an HPLC tracing for human urine before and after the addition of aflatoxins. Resolution and recovery were excellent with no interfering peaks in the B_1 to M_1 region. Figure 4 shows typical results for human serum. There

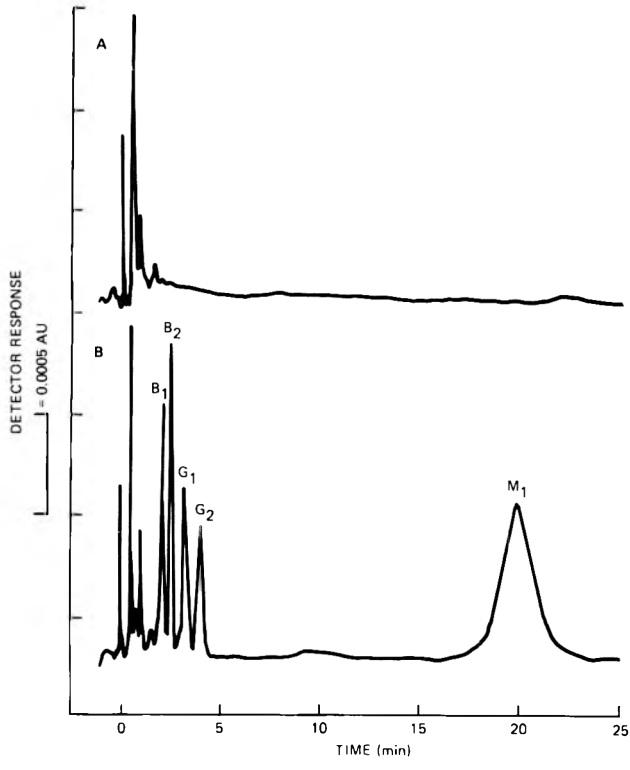


Figure 3. Typical liquid chromatograms of extracted human urine. Unspiked control (A) and aflatoxin spiked (B) at levels of 32 ng B_1/g, 38.4 ng B_2/g, 44.8 ng G_1/g, 38.4 ng G_2/g, and 320 ng M_1/g. Separation on 10 μm silica gel with Pons solution and detection at 365 nm, sensitivity at 0.005 AUFS.

were 2 peaks that eluted 10–30 s before the B_1 peak. They tailed into the B_1 peak and their interference would raise the detection limit for B_1 in serum. Figure 5 shows the representative HPLC scans for the extracted liver sample. There were 2 unidentified peaks that eluted on either side of the G_1 peak, which interfered with the detection of aflatoxins G_1 and G_2. There were no interfering peaks in the B_1 region which affected its detection. A contaminant peak eluted approximately 3 min after the M_1 peak, but caused no problem with detection.

Programmed Flow Rate Analysis

To couple the resolution of aflatoxins B_1, B_2, G_1, and G_2 at slow flow rates with the speed of fast flow rates attained with the radial compression system and desirable in the normal phase elution of aflatoxin M_1, an HPLC analysis using a flow rate change was performed. Samples of the mixed aflatoxin standard, extracted blank, and spiked human liver were analyzed with a dual flow rate program. Representative HPLC scans are shown in Figure 6. The samples were injected at a flow rate of 2 mL/min. At this flow aflatoxins B_1, B_2, G_1, and G_2 eluted between 6 and 14 min. At the 15 min post-injection mark the flow rate was increased to 8 mL/min. Approximately 30 s was required for the baseline to stabilize at which time the chart pen was repositioned at the original baseline. Aflatoxin M_1 then eluted at 26 min. This procedure gave good resolution of all aflatoxin peaks with the exception of G_1, which was still partially overlapped by an interfering peak.

This HPLC method is suitable for the primary analysis of aflatoxins from tissues and biological fluids and for the rapid analysis of a wide range of polar aflatoxin metabolites that are produced by mammalian systems. This is possible because of the pressure characteristics of the radial compression system, which allow flow rate changes to be used as an instrument variable. With this method, retention times are shifted by changing the flow rates.

The ability to make wide changes in flow rate

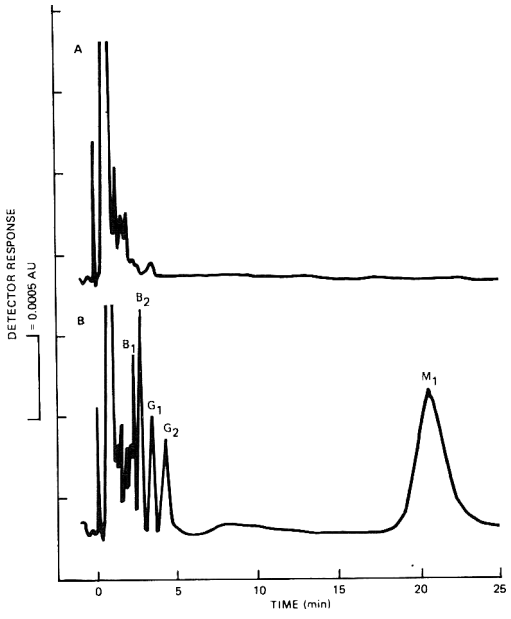


Figure 4. Typical liquid chromatograms of extracted human serum. Unspiked control (A) and aflatoxin spiked (B) at levels of 32 ng B_1/g, 38.4 ng B_2/g, 44.8 ng G_1/g, 38.4 ng G_2/g, and 320 ng M_1/g. Separation on 10 μm silica gel with Pons solution and detection at 365 nm, sensitivity at 0.005 AUFS.

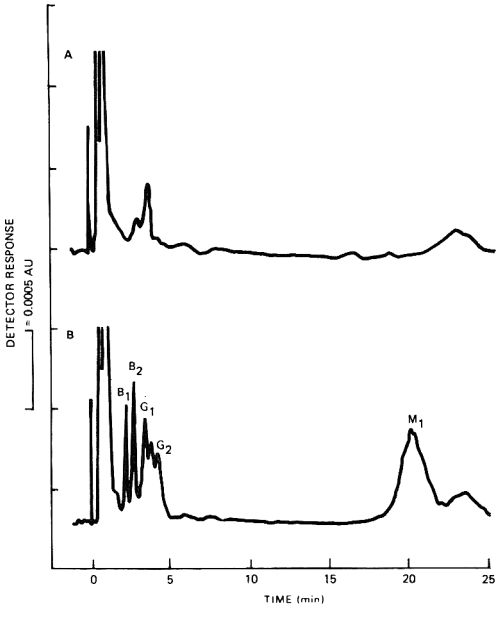


Figure 5. Typical liquid chromatograms of extracted human liver tissue. Unspiked control (A) and aflatoxin spiked (B) at levels of 32 ng B_1/g, 38.4 ng B_2/g, 44.8 ng G_1/g, 38.4 ng G_2/g, and 320 ng M_1/g. Separation on 10 μm silica gel with Pons solution and detection at 365 nm, sensitivity at 0.005 AUFS.

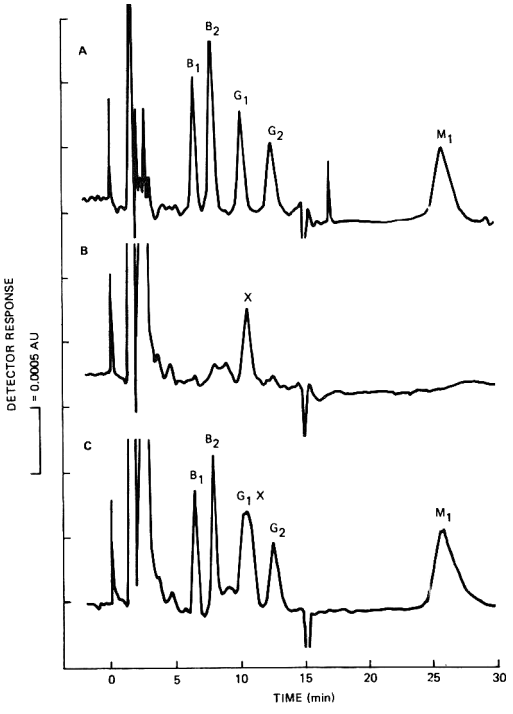


Figure 6. HPLC separation using a programmed flow rate change (2 mL/min for 15 min then changed to 8 mL/min). Aflatoxin standard (A) containing B_1 (20 ng), B_2 (24 ng), G_1 (28 ng), G_2 (24 ng), and M_1 (200 ng). Control liver (B) showing interfering peak (X) at 10.6 min. Human liver sample (C) spiked with mixed aflatoxin standard.

with the radial compression system makes this modification of the Pons method extremely versatile. It allows the flexibility of slow flow rates for maximum detection sensitivity or fast flow rates for shorter analysis times. Dual flow rate separations may also be used, providing the advantages of both.

Failure to evaluate the human health implications of the aflatoxins has resulted frequently from lack of a rapid, sensitive, reliable diagnostic technique for the identification of the aflatoxins and metabolites in tissues and biological fluids. This report describes methods of analysis which do not require extensive analysis time and which may be useful in early detection and clinical diagnosis of aflatoxicosis or aflatoxin-related disease in humans and animals.

REFERENCES

(1) Swenson, D. H. (1979) *Reviews in Biochemical Toxicology*, Vol. 3, Elsevier/North-Holland, New York, NY, pp. 155–192

(2) Newberne, P. M., & Butler, W. H. (1969) *Cancer Res.*

29, 236–250
(3) Ueno, Y., Kubota, K., Ho, T., & Nakamura, Y. (1978) *Cancer Res.* **38**, 536–542
(4) Ciegler, A. (1979) *Ann. N.Y. Acad. Sci.* **329**, 285–292
(5) Wilson, B. J. (1978) *J. Food Protection* **41**, 375–384
(6) Pons, W. A., Jr (1975) *J. Assoc. Off. Anal. Chem.* **58**, 746–753
(7) Pons, W. A., Jr (1976) *J. Assoc. Off. Anal. Chem.* **59**, 101–105
(8) Gregory, J. F., III, & Manley, D. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 144–151
(9) Colley, P. J., & Neal, G. E. (1979) *Anal. Biochem.* **93**, 409–418
(10) Beebe, R. M. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 1347–1352
(11) Siraj, M. Y., et al. (1981) *Toxicol. Appl. Pharmacol.* **58**, 422–430

High Pressure Liquid Chromatographic Determination of Aflatoxins in Peanut Butter Using a Silica Gel-Packed Flowcell for Fluorescence Detection

OCTAVE J. FRANCIS, JR, LEO J. LIPINSKI, JEAN A. GAUL, and ALFRED D. CAMPBELL[1]

Food and Drug Administration, 4298 Elysian Fields Ave, New Orleans, LA 70122

A high pressure liquid chromatographic method has been developed for determining aflatoxins B_1, B_2, G_1, and G_2 in peanut butter. The method is based on extraction with acidified aqueous methanol, partition of the aflatoxins into methylene chloride, and purification of the extract on a 2 g silica gel column. The extracted aflatoxins are resolved on a microparticulate (10 μm) porous silica gel column in ca 10 min with a water-washed chloroform-cyclohexane-acetonitrile solvent that contains 2% isopropanol. The fluorescence detection system determines aflatoxins B_1, B_2, G_1, and G_2 at low levels, i.e., 0.25 ppb B_1, 0.5 ppb G_1, and 0.2 ppb B_2 and G_2. Multiple assays of 5 samples of naturally contaminated peanut butters containing total aflatoxins ($B_1 + B_2 + G_1 + G_2$) at levels of 1, 2, 3, 9, and 17 ppb gave intralaboratory coefficients of variation of 7, 4, 4, 11, and 3%, respectively. Samples spiked at levels of 5, 9, and 17 ppb total aflatoxins showed recoveries of 79, 81, and 81%, respectively.

High pressure liquid chromatography (HPLC) and fluorescence detection are excellent techniques for improving the accuracy and precision of aflatoxin quantitation and are increasingly being incorporated into methods for determining aflatoxins in various agricultural commodities.

Several investigators have used some of the techniques described here for peanut products and corn. Pons and Franz (1) reported that the most consistent results for all types of peanut products were obtained by extracting with methanol-0.1N HCl (4 + 1), followed by partitioning into methylene chloride and subsequent purification of the extract on a 2 g silica gel column. The aflatoxins were detected by HPLC using the same mobile phase described here. The purified aflatoxins B_1 and B_2 were determined by ultraviolet (UV) absorbance at 360–365 nm; G_1 and G_2 were determined by using fluorescence in a silica gel-packed flowthrough cell.

The work reported here combines the extraction and cleanup of Pons and Franz (1) with the packed flowcell fluorescence detection system developed by Panalaks and Scott (2) and later used by Pons (3). This combined procedure eliminates the need for 2 detectors for determining the 4 aflatoxins in peanut butter. It provides for precise and sensitive detection of all toxins at levels as low as 1.0 μg/kg, total, without the need to derivatize B_1 and G_1 to B_{2A} and G_{2A} as is required in reverse phase-fluorescence systems because of the lack of fluorescence of B_1 and G_1 in such systems. The problem of weak solution fluorescence exhibited by B_1 and B_2 in normal phase-fluorescence systems is also eliminated. In this system, fluorescence detection is preferred because it offers greater sensitivity and selectivity than does UV detection

METHOD

Apparatus

(a) *Liquid chromatograph.*—Model ALC/GPC 204 (Waters Associates, Milford, MA 01757) with 6000-A pump and U6K septumless injector; Fluorichrom fluorescence detector (Varian Instrument Division, Palo Alto, CA 94303); 7-54, +7-60 excitation filters (360 nm), 3-73, +4-76 glass emission filters, or 430 nm interference filters, fitted with Varian flowcell packed with LiChrosorb 60 silica gel (30 μm); Spectrum 1021 electronic noise filter, 0.01 Hz cutoff (Spectrum Scientific Corp., Newark, DE 19711); 10 in. recorder with 1 mV input, 0.2 in./min (0.5 cm/min) chart speed.

(b) *Column.*—Waters Associates silica gel (10 μm), 3.9 mm id × 30 cm long. Any other microparticulate column capable of separating aflatoxins in standards with near-baseline resolution can be substituted.

(c) *Cleanup column.*—Econo (Bio-Rad Laboratories, Richmond, CA 94804), 1.0 cm id × 30 cm long, nylon stopcock, polyethylene solvent reservoir, porous 35 μm polyethylene bed support.

(d) *Butt tube.*—2.8 cm id × 22 cm long (No. 92195, Corning Glass Works, Corning, NY 14830).

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

Received September 9, 1981. Accepted November 27, 1981.

0004-5756/82/6503-0672-05$01.00

Reagents

(a) *Solvents.*—Anhydrous ethyl ether (0.05% ethanol), $CHCl_3$, cyclohexane, acetonitrile, and water, distilled in glass and suitable for HPLC (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442).

Caution: $CHCl_3$ is a suspected carcinogen. Use necessary safety measures when handling this chemical.

(b) *HPLC elution solvent.*—Wash 1 L $CHCl_3$ with four 100 mL portions of distilled (HPLC) water, shaking ca 1 min each time in separatory funnel. After fourth wash, let phases separate and let $CHCl_3$ equilibrate overnight Draw off lower $CHCl_3$ layer for use in preparing HPLC elution solvent. Use water-washed $CHCl_3$ only for HPLC.

Prepare HPLC elution solvent by mixing water-washed $CHCl_3$–cyclohexane–acetonitrile (750 + 225 + 30); mix with isopropanol to contain 2% isopropanol. Alcohol content may be varied slightly to adjust retention times.

(c) *Silica gel 60.*—E. Merck (Darmstadt) 0.063–0.2 mm. Activate by drying 1 h at 105°C. Place dried silica gel in air-tight container, add 1 mL water/100 g silica gel, seal container, shake until thoroughly mixed, and store at room temperature >15 h in air-tight container before use.

(d) *Aflatoxin standards.*—In HPLC elution solvent, prepare standard solution containing 0.25 ng each of B_1 and G_1, and 0.075 ng each of B_2 and G_2/mL. Commercial mixed standards (Applied Science Laboratories, Inc., State College, PA 16901, or Supelco, Inc., Bellefonte, PA 16823) are suitable for preparing HPLC standard. Store HPLC standards in threaded vials (5 mL) fitted with size 21 Mininert valve (Kontes Glass Co., Vineland, NJ 08360).

Extraction

Weigh 50 g sample into 1 L blender, add ca 5 g acid-washed diatomaceous earth filter aid, 50 mL 0.1N HCl, and 200 mL methanol, and blend 3 min at high speed. Filter through folded 24 cm Whatman 2V paper, or equivalent, pouring first ca 30 mL filtrate back through filter. Collect ≥50 mL clear filtrate.

Partition

Measure 50 mL filtrate into 250 mL separatory funnel. Add 50 mL 10% NaCl solution and 50 mL hexane (bp 68–69°C) and vigorously shake ca 1 min. Let phases separate and drain lower aqueous layer into another 250 mL separatory funnel. Discard hexane layer. Add 25 mL CH_2Cl_2 and vigorously shake ca 1 min. Let phases separate and drain lower CH_2Cl_2 layer through Butt tube containing ca 4 cm anhydrous granular Na_2SO_4, which has been prewashed with CH_2Cl_2. Collect eluate in 125 mL Phillips beaker. Repeat partition with additional 25 mL CH_2Cl_2 and pass through same Butt tube. Wash Butt tube with ca 25 mL CH_2Cl_2. Add boiling chips and evaporate combined CH_2Cl_2 extracts on steam bath under stream of nitrogen to ca 1 mL.

Column Chromatography

Add anhydrous ethyl ether–hexane (3 + 1) to Econo column until tube is ca one-half full; then add 2 g silica gel. Wash sides of tube with ether–hexane (3 + 1) and stir to disperse silica gel. When rate of settling slows, drain some ether-hexane while gently tapping side of tube to aid settling, leaving 5–7 cm ether-hexane above silica gel. Slowly add ca 1.5 g Na_2SO_4 to top of silica gel. Drain ether-hexane to top of Na_2SO_4.

Table 1. Reproducibility of retention time and peak heights, 11 successive standard injections [a]

Statistic	B_1	B_2	G_1	G_2
Retention time, min [b]				
Range	6.41–6.81	7.35–7.95	8.29–8.90	9.88–10.89
Mean	6.52	7.53	8.51	10.19
Std dev., min	0.14	0.22	0.26	0.38
Coeff. of var., %	2.1	2.9	2.7	3.7
Peak height, mm				
Range	53–60	31–36	48–51	31–35
Mean	56.0	33.1	49.0	33.0
Std dev.	2.72	1.81	1.27	1.15
Coeff. of var., %	4.9	5.5	2.6	3.5

[a] Each injection of aflatoxin standard mixture contained 0.25 ng B_1 and G_1 and 0.075 ng B_2 and G_2.
[b] Measured by electronic integrator.

Table 2. Determination of aflatoxins (μg/kg) in naturally contaminated peanut butter samples

Sample	Statistic	B_1	B_2	G_1	G_2	Total
1	Range	0.62–0.75	0.14–0.17	0.11–0.20	0.11–0.15	1.01–1.24
	Mean[a]	0.71	0.16	0.15	0.13	1.16
	Std dev.	0.05	0.01	0.04	0.02	0.08
	Coeff. of var., %	7.0	7.4	26.7	15.4	6.9
2	Range	13.4–14.3	3.1–3.3	ND[b]	ND	16.5–17.6
	Mean[c]	13.7	3.14	ND	ND	16.8
	Std dev.	0.38	0.08	ND	ND	0.47
	Coeff. of var., %	2.8	2.5	ND	ND	2.8
3	Range	0.90–0.96	0.20–0.21	0.33–0.41	0.13–0.16	1.57–1.71
	Mean[c]	0.92	0.20	0.36	0.15	1.63
	Std dev.	0.03	0.01	0.03	0.01	0.06
	Coeff. of var., %	3.3	5.0	8.3	6.7	3.7
4	Range	1.78–1.88	0.40–0.44	0.54–0.71	0.25–0.28	2.99–3.26
	Mean[c]	1.82	0.42	0.60	0.27	3.11
	Std dev.	0.04	0.02	0.07	0.01	0.12
	Coeff. of var., %	2.2	4.8	11.7	3.7	3.9
5	Range	5.53–6.46	1.07–1.23	1.22–2.01	0.31–0.44	7.62–10.02
	Mean[c]	6.02	1.16	1.72	0.38	9.28
	Std dev.	0.35	0.07	0.31	0.06	0.98
	Coeff. of var., %	5.8	6.0	18.0	15.8	11.0

[a] Average of 6 subsamples, independent assays.
[b] None detected.
[c] Average of 5 subsamples, independent assays.

Dissolve sample extract from partitioning procedure in ca 3 mL CH_2Cl_2 and transfer to column. Wash beaker twice with ca 2 mL CH_2Cl_2, add wash to column, and drain to top of Na_2SO_4. Wash column first with 40 mL benzene–acetic acid (9 + 1), and then with 50 mL ether–hexane (3 + 1), draining each wash to top of Na_2SO_4. Discard washes. Elute aflatoxins with 60 mL CH_2Cl_2–acetone (9 + 1), and collect eluate in 125 mL Phillips beaker. Add boiling chips and evaporate to near dryness on steam bath under stream of nitrogen.

If guard column is used in HPLC system, quantitatively transfer sample to 5 mL screw-cap vial, using several small volumes of CH_2Cl_2. Evaporate solvent to dryness under stream of nitrogen, and seal vial with foil-backed or Teflon-lined screw cap. Reserve for HPLC. Sample can be stored as dry film until ready for use.

If no guard column is used in HPLC system, dissolve extract in ca 1 mL CH_2Cl_2; pour through 2 mL Buchner funnel fitted with coarse porosity fritted disk overlaid with tightly pressed circle of Whatman GF/A glass fiber paper slightly larger than fritted disk (No. 9 cork borer). Collect in 5 mL screw-cap vial. Wash beaker with 3 ca 1 mL portions of CH_2Cl_2, pouring washes through funnel to remove particulate matter, which could plug HPLC column filter, and collect in vial. Evaporate to dryness under stream of nitrogen, and seal with foil-backed or Teflon-lined screw cap. Reserve for HPLC. Sample can be stored as dry film until ready for use.

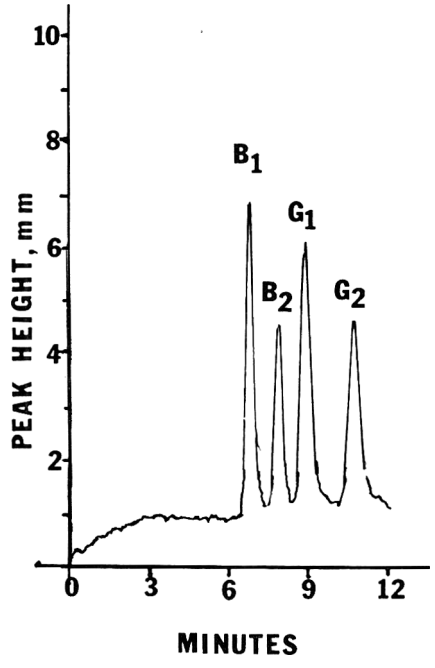


Figure 1. Chromatogram of standard solution of B_1 and G_1, 0.25 ng, and B_2 and G_2, 0.075 ng. Fluorescence detection, silica gel-packed column; 10 μL injection of aflatoxin standard solution.

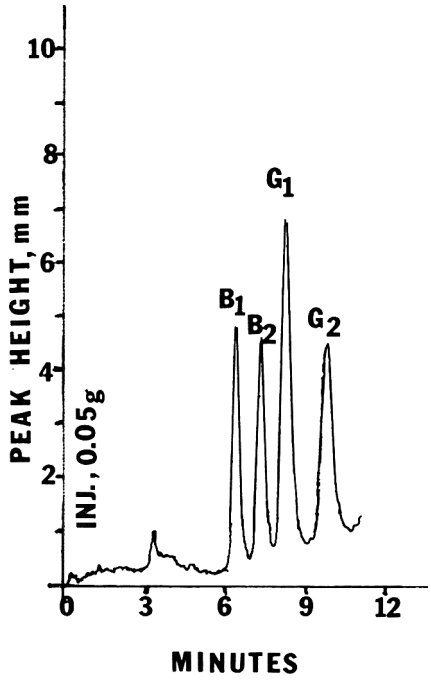


Figure 2. Chromatogram of naturally contaminated peanut butter sample fortified to contain 17 μg total aflatoxins B_1, B_2, G_1, and G_2/kg. Fluorescence detection, silica gel-packed column; 10 μL injection.

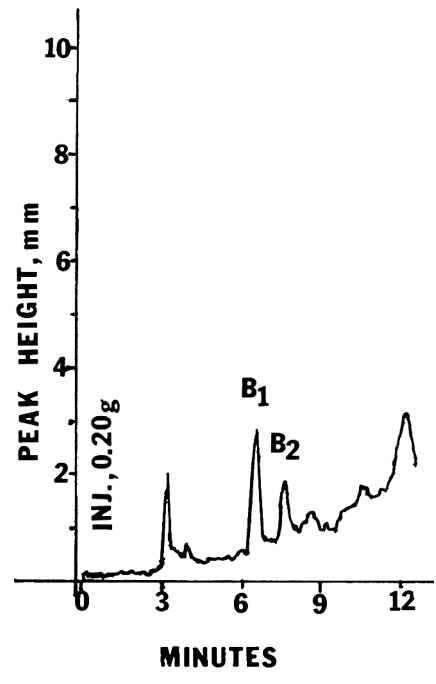


Figure 3. Chromatogram of naturally contaminated peanut butter sample. Fluorescence detection, silica gel-packed column; 10 μL injection.

High Pressure Liquid Chromatography

Set up detector and HPLC apparatus, using high lamp, high gain, and attenuation of 20 or 50×. Stabilize HPLC at flow rate of ca 1.0 mL/min. Flow rate can be adjusted for best resolution of aflatoxins. Baseline noise should be <0.5%.

Inject 10 μL standard solution; peaks should be off scale. Dilute aliquot of standard solution with HPLC solvent so that 10–20 μL injection yields B_1 peak ca 50% full-scale deflection (FSD). Usually 0.3 ng B_1 gives ca 50% FSD. Use this standard as working standard. Use standard dilution factor for sample dilution. Inject individual aflatoxin standards to determine order of aflatoxin elution. Order of elution by this system is B_1, B_2, G_1, G_2.

Sample Dilution

Sample dilution is dependent on sensitivity of packed cell and individual fluorescent detector, and is also determined by desired screening level. Optimum instrumental conditions permit detection of 0.25 ppb B_1 (10% FSD), 0.5 ppb G_1, and 0.2 ppb B_2 and G_2.

Dissolve sample extract in 500 μL HPLC solvent for levels of B_1 ranging from minimum detectable level to 2.5 ppb for 10 μL injection. Higher screening levels will necessitate further dilutions to adjust sample concentration to approximate peak area or height of standard. Inject 10 μL sample extract and record chromatogram beyond time for elution of G_2.

Following HPLC elution of aflatoxins in samples, flush system with mobile solvent 15 min at ca 4 mL/min to remove crop co-extractants that follow aflatoxins and which will interfere with next injection.

From either electronic integrator area, or peak height (mm) measured from baseline to apex of B_1 peak, calculate B_1 content.

$$B_1, \text{ng/g} = (A_x \times C_s \times V_s \times D)/(A_s \times V_x \times W)$$

where A_x = area count, or peak height, of B_1 sample peak; C_s = concentration of B_1 in standard, ng/μL; V_s = μL standard injected; D = dilution volume of sample extract, μL; A_s = area count, or peak height of B_1 standard peak; V_x = μL sample extract injected; W = g sample represented by sample extract, 10 g. For other aflatoxins, substitute appropriate concentrations, areas, or peak heights.

Results and Discussion

The quantitative reproducibility of the 4 aflatoxin standards contained in a mixture was determined by repetitive injections at constant volume and concentration over a 6-h period. Sample extracts were injected between standard injections. Measured retention times, min, were 6.52 (B_1), 7.53 (B_2), 8.51 (G_1), and 10.19 (G_2), with coefficients of variation ranging from 2.2 to 3.7%. Results are shown in Table 1. Reproducibility of aflatoxin peak heights is also shown in Table 1. Linearity studies of the 4 aflatoxins showed each to be linear for the concentrations tested (B_1 and G_1, 0.090–0.750 ng and B_2 and G_2, 0.025–0.225 ng); correlation coefficients (r) of 0.9998, 1.000, 0.9992, and 0.9999 were obtained for aflatoxins B_1, B_2, G_1, and G_2, respectively.

To find an aflatoxin-free peanut butter sample, 5 different samples were analyzed. Samples 1, 3, and 4, which were negative for aflatoxins by the CB method (4), were examined. None of those samples were negative for aflatoxins when analyzed by the method reported here. Sample 1 (Table 2) was the least contaminated of all those examined and subsequently was selected for use in the recovery study.

Six portions of a naturally contaminated peanut butter (Sample 1) were fortified in duplicate at three levels, with total aflatoxins B_1, B_2, G_1, and G_2. The percentages of aflatoxins recovered at the 5 ppb level were: B_1, 78, 84; B_2, 83, 85; G_1, 76, 77; G_2, 71, 75; total, 77, 80. At the 9 ppb level, the percentages were: B_1, 82, 85; B_2, 85, 87; G_1, 77, 80; G_2, 71, 76; total, 79, 82. At the 17 ppb level, the percentages were: B_1, 83, 80; B_2, 86, 84; G_1, 79, 79; G_2, 77, 77; total, 81, 80.

Aflatoxins B_1, B_2, G_1, and G_2 were completely resolved in chromatograms of standards, fortified samples, and naturally contaminated peanut butter extracts, as shown in Figures 1–3. In the desired detection area, samples were essentially free of the interference frequently caused by crop co-extractants.

Acknowledgments

The authors thank Arthur E. Waltking, CPC International, Union, NJ and Stepher E. Rayner, U.S. Department of Agriculture, Washington, DC for supplying samples.

References

(1) Pons, W. A., Jr, & Franz, A. O., Jr (1978) *J. Assoc. Off. Anal. Chem.* **61**, 793–800
(2) Panalaks, T., & Scott, P. M. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 583–589
(3) Pons, W. A., Jr (1979) *J. Assoc. Off. Anal. Chem.* 62, 586–594
(4) *Official Methods of Analysis* (1980) 13th Ed. AOAC, Arlington, VA, secs **26.026–26.036**

PESTICIDE RESIDUES

Simple, Low-Cost Method for Determination of Selected Chlorinated Pesticides in Fat Samples

STEFAN M. WALISZEWSKI and GRZEGORZ A. SZYMCZYŃSKI[1]
Institute of Plant Protection, Poznań, Poland

A method is described for the quantitative and qualitative determination of selected chlorinated pesticides in fat samples. Pesticide residues are extracted with petroleum ether and separated from fat with concentrated H_2SO_4 instead of the commonly used adsorbents Florisil, alumina, or silica gel. Residues were analyzed by gas chromatography with electron capture detection. Recoveries of fortified samples were approximately 100%.

Many different methods are used to determine residues of persistent chlorinated pesticides in samples with high fat content. The pesticides are separated from fat by column chromatography on Florisil (1–6), Al_2O_3 (7–11), or silica (12–16), by sweep-codistillation (17–20), and by extraction with concentrated H_2SO_4 (21–23). The latter method is suitable only for pesticides stable to concentrated H_2SO_4. The methods specifying fat adsorption are expensive because of the relatively high costs of these adsorbents. Solvent partitioning requires large quantities of reagents, and sweep-codistillation is a labor-intensive technique. Thus, we focused on concentrated H_2SO_4 as a means of extracting persistent chlorinated insecticides.

On the basis of the experience of other researchers (21–23) and our own experience (24, 25), we wished to develop a simple, low-cost method for determination of persistent chlorinated pesticides in human fat tissue. However, our samples of human adipose tissue had been collected during post-mortem examinations and during therapeutic surgery (25) and were highly contaminated. Therefore, recoveries for the method were determined on butter fat.

Experimental

Reagents and Apparatus

(a) *Petroleum ether.*—bp 40–50°C.

(b) *Gas chromatograph.*—Varian Model 2100 with ^{63}Ni electron capture detector and glass, U-shaped column, 360 cm × 2 mm, packed with 1.5% OV-17 + 1.95% OV-210 on 80–100 mesh Gas-Chrom Q. *Operating conditions:* nitrogen carrier gas at 30 mL/min; column 185°C; detector 210°C; injector 250°C.

(c) *Evaporator.*—Büchi (Switzerland).

Extraction and Cleanup

The homogenized fat sample was ground with enough anhydrous Na_2SO_4 to form a coarse powder. The sample was transferred to a chromatographic column and residues were eluted with 150 mL petroleum ether. The eluate was evaporated at 38–40°C in a rotary evaporator to reduce fat concentration to ca 500 mg/10 mL. The exact fat content in the concentrated extract was determined gravimetrically.

An aliquot containing ≤500 mg fat was pipetted into a 10 mL calibrated tube with a cut-glass stopper. The volume was adjusted to 10.0 mL with petroleum ether. Then 0.5 mL concentrated H_2SO_4 was added and the tube was tightly stoppered and vigorously shaken ca 20 s. The tube was left for 1 min; 5 mL supernate was removed by pipet and dried by passing through 3–5 g Na_2SO_4. The Na_2SO_4 was washed with petroleum ether and the ether extract was evaporated to 0.1 mL and quantitatively transferred to a 1.0 mL volumetric tube. The volume was adjusted and an aliquot was injected into the gas chromatographic column.

Pesticides were determined quantitatively by both peak height and peak area.

Discussion

Table 1 presents results for 237 mg butter fat with the following chlorinated pesticides: HCB, α-BHC, γ-BHC, β-BHC, δ-BHC, ϵ-BHC, heptachlor epoxide, DDE, Kepone, *o,p'*-DDT, *p,p'*-DDT, and mirex. The recoveries (n = 10) showed good repeatability and ranged from 89 to 104% except 76.9% for δ-BHC and 47.9% for Kepone. These low recoveries were caused by increased solubility of these pesticides in the fat–H_2SO_4 phase (unpublished data).

[1] Department of Andrology, Medical School of Poznań, Jacowskiego 41, PL-60-513 Poznań, Poland. Address correspondence to this author.

Received May 8, 1981. Accepted October 14, 1981.

0004-5756/82/6503-0677–03$01.00

Table 1. Fortification levels and recoveries of selected chlorinated pesticides in 237 mg butter fat

Compound	Fortification level, ppm	Mean recovery, %[a]	SD	CV, %
HCB	0.090	95.9	6.6	6.9
α-BHC	0.090	96.6	9.4	9.7
γ-BHC	0.190	100.4	7.7	7.7
β-BHC	0.180	97.8	4.4	4.5
δ-BHC	0.186	76.9	5.0	6.6
ϵ-BHC	0.182	90.1	4.0	4.4
Heptachlor epoxide	0.198	88.7	4.6	5.2
DDE	0.202	101.5	6.8	6.7
Kepone	1.450	47.9	6.0	12.5
o,p'-DDT	0.532	104.3	7.0	6.7
p,p'-DDT	0.758	98.4	11.6	11.8
Mirex	1.072	96.4	6.7	7.0

[a] $n = 10$.

Good extraction of fat was observed with 0.5 mL concentrated H_2SO_4, and the dry residue was never higher than 1% of the initial fat content of a sample. About 20 times less H_2SO_4 is used in this method than that reported by Veierov and Aharonson (21, 22).

Extracting a larger amount of fat, up to 1.5 g, did not increase the percentage of nonvolatile

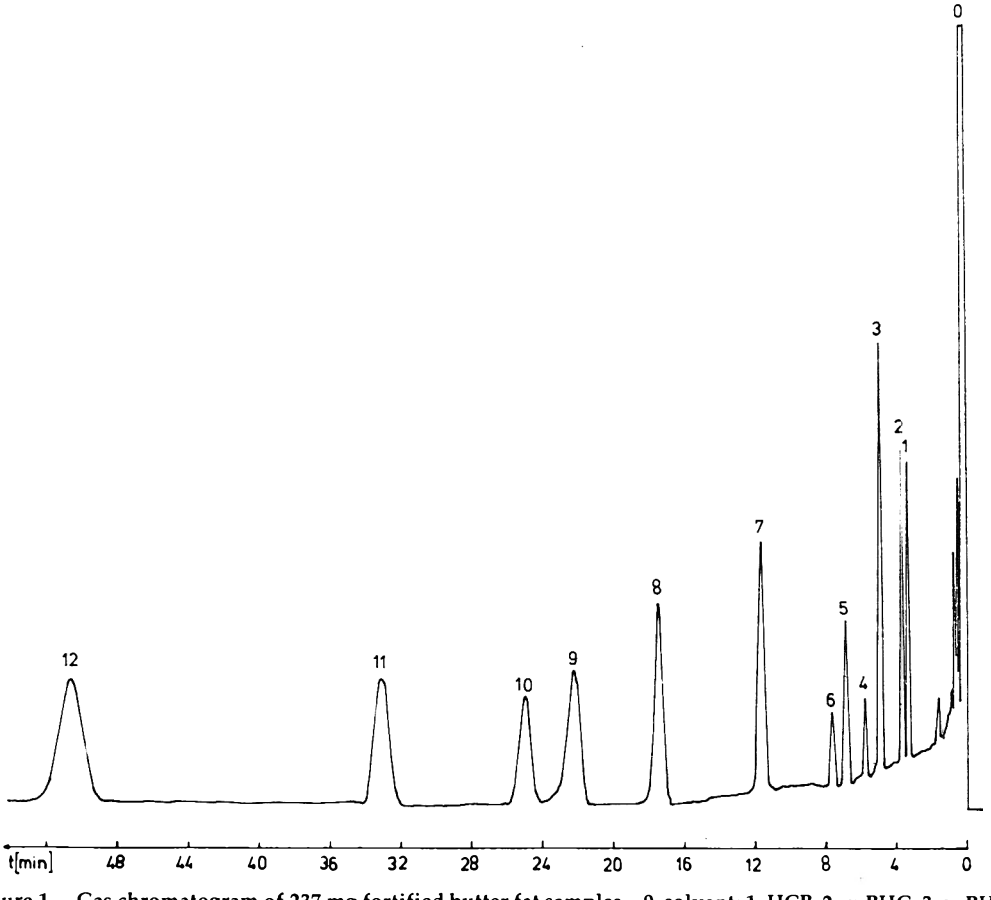


Figure 1. Gas chromatogram of 237 mg fortified butter fat samples. 0, solvent; 1, HCB; 2, α-BHC; 3, γ-BHC; 4, β-BHC; 5, δ-BHC; 6, ϵ-BHC; 7, heptachlor epoxide; 8, DDE; 9, Kepone; 10, *o,p'*-DDT; 11, *p,p'*-DDT; 12, mirex.

residues. Instead, recoveries decreased due to the increased solubility in the fat-H_2SO_4 phase.

A chromatogram of a sample of butter fat fortified with the amounts of chlorinated pesticides shown in Table 1 is presented in Figure 1. The method allows for simple and inexpensive routine monitoring of samples with a high fat content for chlorinated pesticides contamination, although it cannot be recommended for determining Kepone. The cost of analysis is lowered significantly by replacing 30 g expensive adsorbent by 0.5 mL concentrated H_2SO_4. To increase the sensitivity of the analytical method, 2 extracts of the same sample may be combined, evaporated to 1.0 mL, and determined by GLC.

References

(1) Mills, P. A., Bong, B. A., Kamps, L. R., & Burke, J. A. (1972) *J. Assoc. Off. Anal. Chem.* **55,** 39–43

(2) Stijve, T., & Cardinale, E. (1974) *Mitt. Geb. Lebensmittelunters Hyg.* **65,** 131–150

(3) Stijve, T., & Brand, E. (1977) *Dtsch. Lebensm. Rundsch.* **2,** 42–43

(4) Suzuki, T., Ishikawa, K., Sato, N., and Sakai, K.-I. (1979). *J. Assoc. Off. Anal. Chem.* **62,** 689–694

(5) Stimac, R. M. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 85–88

(6) Stijve, T. (1976) *Rüchstandsanalyltik von Pflazenschutzmitteln*, Vol. 4, Lieferung, s. 9, pp. 1–13

(7) Telling, G. M., & Sisons, D. J. (1977) *J. Chromatogr.* **137,** 405–423

(8) Steinwandter, H., & Schlüter, H. (1979) *Dtsch. Lebensm. Rundsch.* **5,** 141–148

(9) Steinwandter, H., & Bass, H. (1975) *Chemosphere* **1,** 27–30

(10) Greve, P. A., & Grevenstuk, W. B. F. (1975) IUPAC Third International Congress of Pesticide Chemistry, Helsinki, 3–9 July 1974, Georg Thieme Publishers, Stuttgart, GFR

(11) Wells, D. E., & Johnstone, S. J. (1977) *J. Chromatogr.* **140,** 17–28

(12) Steinwandter, H., & Schlüter, H. (1978) *Dtsch. Lebensm. Rundsch.* **4,** 139–141

(13) Steinwandter, H., & Schlüter, H. (1977) *Z. Anal. Chem.* **286,** 90–94

(14) Fuchsbichler, G. (1979) *Landwirtsch. Forsch.* **32,** 341–354

(15) Johnson, L. D., Waltz, R. H., Ussary, J. P., & Kaiser, F. E. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 174–187

(16) Specht, W., & Tillkes, M. (1980) *Fresenius Z. Anal. Chem.* **301,** 300–307

(17) Heath, A., & Black, R. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 757–763

(18) Heath, A., & Black, R. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 529–531

(19) Pflugmacher, J., & Ebing, W. (1979) *Z. Lebensm. Unters. Forsch.* **169,** 343–345

(20) Pflugmacher, J., & Ebing, W. (1979) *Landwirtsch. Forsch.* **32,** 82–87

(21) Veierov, D., & Aharonson, N. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 253–260

(22) Veierov, D., & Aharonson, N. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 202–207

(23) Murphy, P. G. (1972) *J. Assoc. Off. Anal. Chem.* **55,** 1360–1362

(24) Waliszewski, S. M., & Szymczyński, G. A. (1981) *Fresenius Z. Anal.Chem.*, in press

(25) Szymczyński, G. A., & Waliszewski, S. M. (1981) *Andrologia* **13**(3), 250–252

Terbufos and Its Metabolites: Identification by Gas-Liquid Chromatography and Mass Spectrometry

LESTER Y. WEI and ALLAN S. FELSOT
Illinois Natural History Survey, Section of Economic Entomology, Champaign, IL 61820

Methods have been developed for identification of terbufos, terbufos sulfoxide, terbufos sulfone, terbufoxon, terbufoxon sulfoxide, and terbufoxon sulfone by mass spectrometry. Residues of terbufos and terbufos sulfone fortified in soil samples at 5 ppm were extracted and analyzed successfully by gas chromatography and mass spectroscopy.

Terbufos (Counter®), *S*-((1,1-dimethylethylthio)methyl) *O,O*-diethyl phosphorodithioate, has been used as a soil insecticide throughout the Midwest since 1975 for control of corn rootworms. Approximately 4 million pounds of technical terbufos were applied on corn acreage in both Illinois and Iowa in 1980. Terbufos is extremely toxic (LD_{50} acute oral, male mouse = 3.5 mg/kg) and is enzymatically converted in biological systems to its phosphorothioate form (terbufoxon) and thioether oxygen homologs (1, 2), terbufos sulfoxide, terbufoxon sulfoxide, terbufos sulfone, and terbufoxon sulfone (Figure 1).

The major metabolites of terbufos in soil have been identified by thin layer radiochromatography as terbufos sulfoxide and terbufos sulfone (3). Column chromatography and gas chromatography (GC) were used by Chapman and Harris (4) to study the persistence of terbufos, terbufos sulfoxide, and terbufos sulfone in soil. Although GC-mass spectrometry (MS) methods are now routinely used for pesticide residue analysis and confirmation (5), the methodology for the analysis of terbufos and its oxidative metabolites has not been published. The objective of our study was to identify terbufos residues in soil by gas chromatography and mass spectrometry.

Experimental

Instrumentation

Low resolution spectra were recorded on a Varian MAT CH-7 mass spectrometer under the following conditions: electron energy, 70 eV; emission current, 300 μA; ion source temperature, 200°C. The mass spectra were recorded on a Spectrosystem 100. A glass column (0.7 m × 3 mm id) packed with 1% EGSS-X on Gas-Chrom Q was used in the Varian Model 1700 interfaced to the mass spectrometer. Temperatures used: oven, 135–170°C (programming rate 10°/min); injection port, 200°C; He flow rate 40 mL/min. Chemical ionization mass spectra were recorded with a Varian MAT 311A instrument (Department of Chemistry, University of Illinois).

For GLC detection, a Varian Model 1400 gas chromatograph equipped with an alkali flame ionization detector was operated at conditions identical to those used in GC-MS analysis.

Materials and Method of Extraction

Terbufos, terbufos sulfoxide, terbufos sulfone, terbufoxon, terbufoxon sulfoxide, and terbufoxon sulfone were obtained from the American Cyanamid Co., Princeton, NJ.

Terbufos and oxidative metabolites were extracted from soil by mixing 45 min with hexane–acetone (2 + 1 v/v, 2 mL solvent/1 g soil). The solvent was filtered through anhydrous Na_2SO_4. The soil residue was re-extracted and the combined filtrate was concentrated on a steam bath by using a Snyder column. Extracts were not concentrated to dryness because terbufos is volatile.

Individual aliquots of a Flanagan silty loam soil (3.5% organic carbon) and a Gilford–Hooperston–Ade sandy loam soil (1.2% organic carbon) were fortified at 5 μg/g (5 ppm) with terbufos or its oxidative metabolites in acetone. After the acetone evaporated, these soils were extracted as described above to determine extraction efficiency. Composite soil samples consisting of eight 2.5 × 15 cm cores taken from a corn plot (Fayette silty loam, 0.9% organic carbon) 5 days after treatment at 1 lb AI/acre were also extracted and analyzed by gas chromatography and mass spectrometry.

Results and Discussion

Extraction Efficiency of Terbufos and Its Metabolites from Soil

All extracts from fortified samples were analyzed directly by gas chromatography except terbufos sulfoxide and terbufoxon sulfoxide. These sulfoxides could not be analyzed directly

Received May 13, 1981. Accepted November 4, 1981.

0004-5756/82/6503-0680-05$01.00

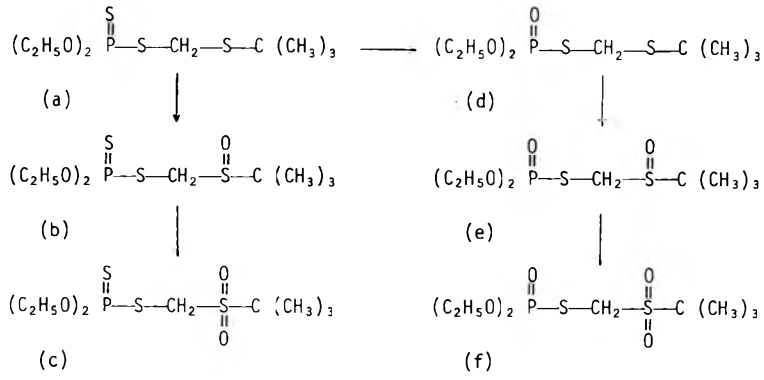


Figure 1. Molecular structures of terbufos and oxidative metabolites: (a) terbufos; (b) terbufos sulfoxide; (c) terbufos sulfone; (d) terbufoxon; (e) terbufoxon sulfoxide; (f) terbufoxon sulfone.

because of their instability on the heated GLC column. This phenomenon has apparently been observed by others (1, 4).

Terbufos sulfoxide and terbufoxon sulfoxide had to be oxidized to the more stable sulfones before GLC analysis. The terbufos sulfoxide extract was oxidized to terbufos sulfone by reaction with potassium permanganate (4). The terbufoxon sulfoxide extract was oxidized to the terbufoxon sulfone by oxidation with metachloroperbenzoic acid (1). These sulfones were then determined by GLC.

Percentage recoveries of terbufos and its metabolites from fortified samples were at least 81% except for the terbufoxon sulfoxide from the Flanagan silty loam (Table 1).

GLC and MS Characterization of Terbufos and Its Metabolites

Terbufos, terbufos sulfone, terbufoxon, and terbufoxon sulfone were individually analyzed by GLC-MS. Their retention time data are given in Tables 2 and 3. Terbufos sulfoxide and terbufoxon sulfoxide were introduced by direct probe because these 2 compounds could not be analyzed directly by GLC. The common fragment ions and the characteristic fragment ions of terbufos, terbufos sulfoxide, and terbufos sulfone are given in Table 2.

An examination of the molecular ions in Table 2 indicates that the parent peak of terbufos is much stronger than those of the sulfoxide or sulfone. One possible correlation between molecular ion abundance and structure of these 3 compounds might relate to the degree of crowding between the bulky tert-butyl group

Table 1. Recovery (%) of terbufos and its metabolites from soil samples fortified at 5 ppm

Compound	Flanagan silty loam soil	Gilford–Hooperston–Ade sandy loam soil
Terbufos	82	84
Terbufos sulfoxide	92	9[illegible]
Terbufos sulfone	89	97
Terbufoxon	86	8[illegible]
Terbufoxon sulfoxide	68	85
Terbufoxon sulfone	85	86

Table 2. The 70 eV mass spectra and GLC retention time of terbufos, terbufos sulfoxide, and terbufos sulfone

	Rel. intensity, %		
m/e	Terbufos	Terbufos sulfoxide	Terbufos sulfone
	Common Ions		
57	100	100	100
65	25	47	29
93	11	31	14
97	44	67	76
121	16	28	20
125	22	60	54
153	30	66	76
186	21	50	4
	Characteristic Ions		
199	—	48	37
231	69	—	—
248	—	42	—
264	—	—	19
M^+	288 (17)	304 (1.2)	320 (1.3)
RT[a] (min)	2.5	—	13

[a] Initial column temperature was 135°C, programmed at 10°/min up to 170°C.

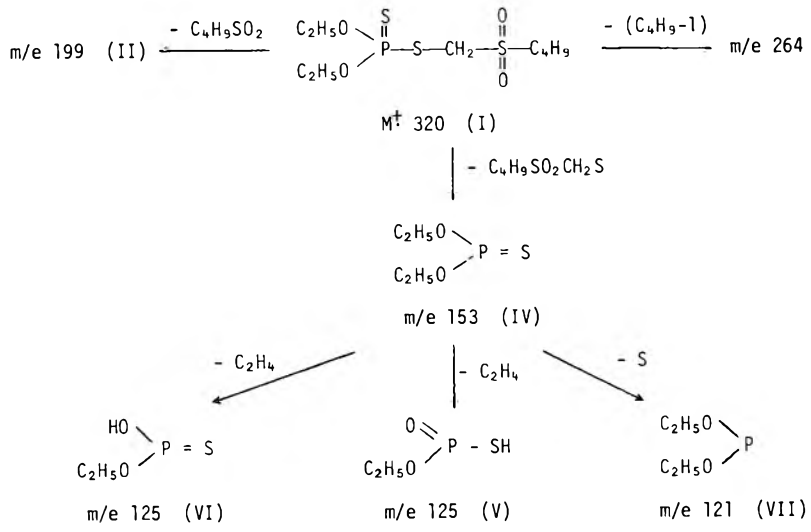


Figure 2. Fragmentation pathways of terbufos sulfone.

and thio, sulfinyl, and sulfonyl groups, respectively (Figure 1). The abundance of ion m/e 199 (Figure 2) from the sulfoxide and sulfone also reflects the steric hindrance of the tert-butyl group.

The common fragment ions and the characteristic fragment ions of terbufoxon, terbufoxon sulfoxide, and terbufoxon sulfone are shown in Table 3. The ions at m/e 81, 109, 137, 170, 183, 215, 232, and 248 are analogous to those at m/e 97, 125, 153, 186, 199, 231, 248, and 264 in Table 2.

The major fragmentation pathways of terbufos sulfone are given in Figure 2. Similar pathways

Table 3. The 70 eV mass spectra and GLC retention time of terbufoxon, terbufoxon sulfoxide, and terbufoxon sulfone

m/e	Rel. intensity, % Terbufoxon	Terbufoxon sulfoxide	Terbufoxon sulfone
	Common Ions		
57	100	100	100
65	17	52	44
81	35	82	65
93	11	48	35
109	23	95	73
137	9	56	36
170	54	101	4
	Characteristic Ions		
183	—	60	61
215	51	—	—
232	—	75	—
248	—	—	20
M^+	272 (31)	—	—
RT[a] (min)	3.5	—	15

[a] Initial column temperature was 135°C, programmed at 10°/min up to 170°C.

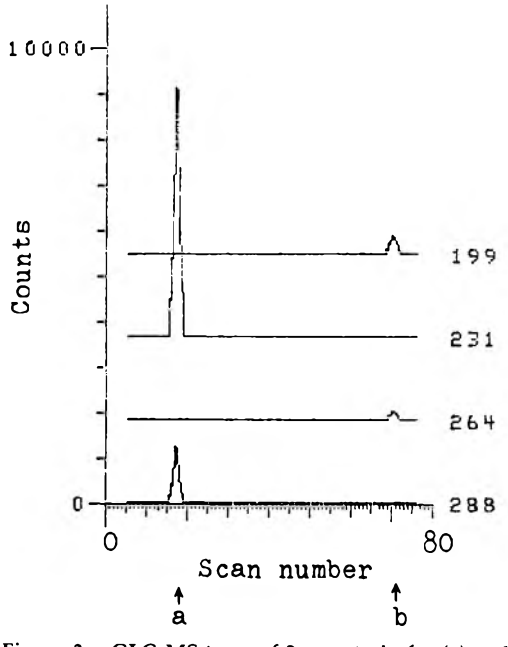


Figure 3. GLC-MS trace of 2 ppm terbufos (a) and 0.3 ppm terbufos sulfone (b).

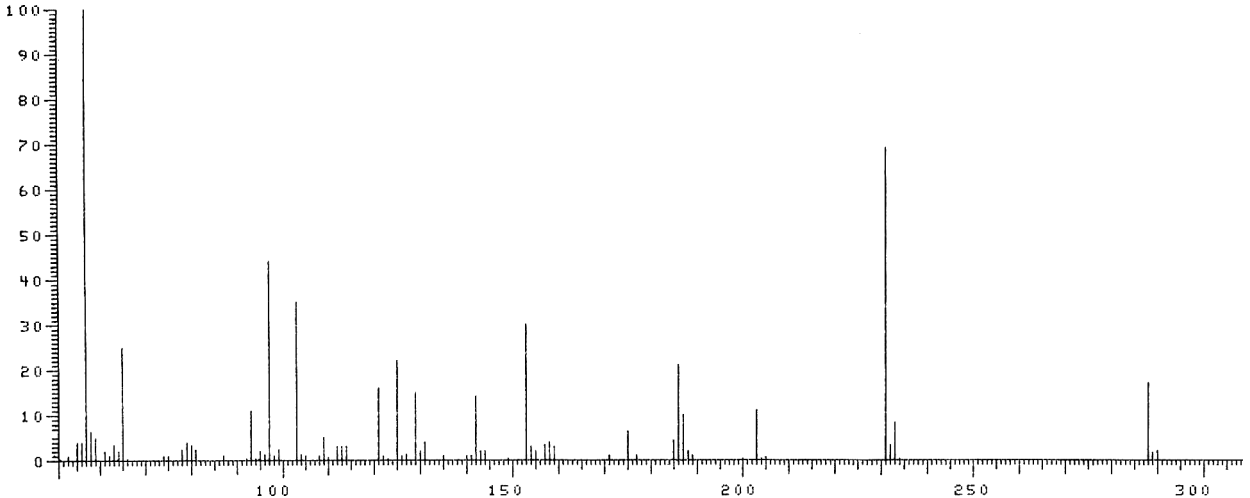


Figure 4. Mass spectrum of terbufos.

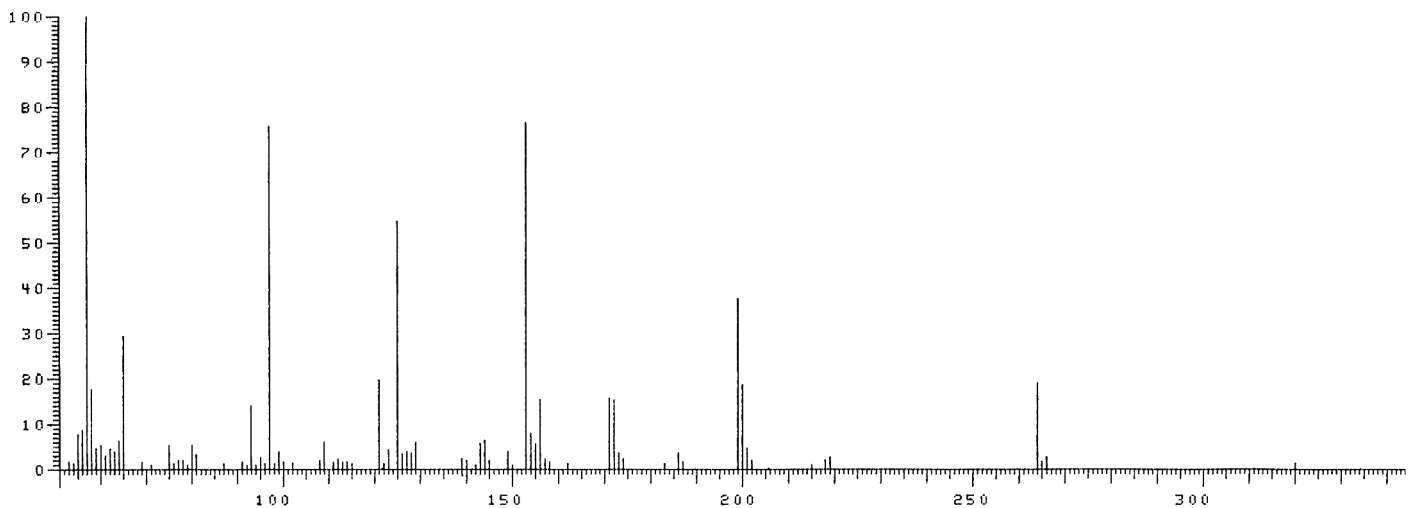


Figure 5. Mass spectrum of terbufos sulfone.

can be envisaged to explain the major ions present in the spectra of the other 5 compounds. Figure 2 shows that the intense peaks at m/e 125 are a composite peak made up of 2 ions (V, VI): the fragment ion produced by McLafferty rearrangement (6) and the fragment ion resulting from the loss of C_2H_4 from the ethoxy group of ions m/e 153 (IV).

Terbufos and its metabolites were also examined by chemical ionization mass spectrometry. The CI spectra showed intense $(M + H)^+$ ions when isobutane was used as a reaction gas.

Identification of Terbufos and Terbufos Sulfone in Soil Samples

Insecticide residues extracted from the field soil were analyzed by GLC-MS. Ions were monitored at m/e 199, 231, 264, and 288. A typical GLC-MS chromatogram of terbufos and terbufos sulfone is shown in Figure 3. Their spectra are given in Figures 4 and 5. Other major fragment ions of terbufos and terbufos sulfone may also be suitable for monitoring if the coextractive of a soil sample interfered in the analysis for ions at m/e 199, 231, 264, and 288. Terbufoxon and terbufoxon sulfone were not observed in soil samples and, as indicated previously, the sulfoxides could not be detected directly by GLC-MS.

Separate solutions of terbufos and terbufos sulfone with concentrations ranging from 1 to 100 ppm were analyzed. The calibration curves generated were linear and indicated that GLC-MS could be used for quantitative analysis.

Previous quantitative analyses of terbufos residues in soil or insects have involved thin layer radiochromatography and column chromatography (2–4). The advantage of our method is that it requires only a simple extraction and is free from interference. It is probably applicable to the analysis of derivatized sulfoxides in soil samples. This possibility of direct GLC-MS detection is being explored.

Acknowledgments

We thank Carter Cook and Richard Milberg (University of Illinois) for chemical ionization mass spectra and their suggestions. This research was supported by the Illinois Natural History Survey and Illinois Agricultural Experiment Station, College of Agriculture, University of Illinois at Urbana-Champaign.

References

(1) Zweig, G. (1980) *Analytical Methods for Pesticides and Plant Growth Regulators*, Vol. 11, Academic Press, Inc., New York, NY, pp. 165–183
(2) Chio, H., & Metcalf, R. L. (1979) *J. Econ. Entomol.* **72**, 732–738
(3) Leveglia, J., & Dahm, P. A. (1975) *Environ. Entomol.* **4**, 715–718
(4) Chapman, R. A., & Harris, C. R. (1980) *J. Econ. Entomol.* **73**, 536–543
(5) Vander Velde, G., & Ryan, J. F. (1975) *J. Chromatogr. Sci.* **13**, 322–329
(6) Damico, J. N. (1966) *J. Assoc. Off. Anal. Chem.* **49**, 1027–1045

Application of the AOAC Multi-Residue Method to Determination of Synthetic Pyrethroid Residues in Celery and Animal Products

HEINZ E. BRAUN and JANA STANEK
Provincial Pesticide Residue Testing Laboratory, Ontario Ministry of Agriculture and Food, Guelph, Ontario, Canada NIG 2W1

A procedure based on current multi-residue methodology is described for the determination of synthetic pyrethroid residues in vegetable and animal tissues. Permethrin, cypermethrin, and fenvalerate are extracted from celery, egg yolk, beef muscle, and milk with acetonitrile, partitioned into hexane, and cleaned up on Florisil for quantitation by electron capture gas chromatography. Recoveries of the 3 pyrethroids averaged 94-103% from celery and 82-97% from animal products. Minimum detectable levels of <5 ng/g are readily attainable. The method also allows for the simultaneous extraction and cleanup of the organochlorine hydrocarbon insecticides which have been routinely analyzed in the past.

The synthetic pyrethroids have become a commercially successful group of insecticides because of their desirable environmental properties of short persistence and nontoxicity to mammals. These features, combined with their broad spectrum of insecticidal activity and the comparatively low application rates required for insect control, have made the pyrethroids environmentally safe and acceptable alternatives to the older organochlorine compounds such as the diphenylethanes and cyclodienes. Permethrin, cypermethrin, and fenvalerate are 3 synthetic pyrethroids which are coming into increased use for insect control on domestic animals and field crops; this report describes an analytical procedure for the determination of these compounds at the residue level in plant and animal tissues. Structural formulas for permethrin, cypermethrin, and fevalerate are illustrated in Figure 1. With the expectation that registered uses of the pyrethroids in food production will become more general, it may be necessary for the residue analyst to include these compounds in monitoring and regulatory programs.

Permethrin, (3-phenoxyphenyl)methyl *cis,-trans*-(±)-3-(2,2-dichloroethyl)-2,2-dimethylcyclopropanecarboxylate, has been described in *Synthetic Pyrethroids* (1). The active ingredients in formulations (e.g., Ambush®, Ectiban®) are the cis and trans isomers which are present in an approximate ratio of 2:3, respectively. Williams (2) reported an analytical procedure for determination of permethrin in potato tubers. Fujie and Fulmer (3) described the determination of permethrin residues in plant, animal, and soil matrices by using various extraction media and quantitating the individual cis and trans isomers by gas chromatography with either electron capture or electrolytic conductivity detection. More recently, Chiba (4) reported on the determination of permethrin isomers on peach foliage. A method for the determination of cypermethrin, α-cyano-3-phenoxybenzyl 2,2-dimethyl-3(2,2-dichlorovinyl) cyclopropanecarboxylate (Ripcord®, Barricade®), in various crops has been described by Chapman and Harris (5).

Fenvalerate, cyano(3-phenoxyphenyl)methyl 3-chloro-α-(1-methylethyl)2-(4-chlorophenyl)-3-benzeneacetate (Pydrin®, Sumicidin®), is a noncyclopropanecarboxylate pyrethroid which has been shown to be effective against several vegetable insect pests. Lee et al. (6) described the determination of fenvalerate in cabbage and lettuce by using acetonitrile extraction, cleanup on partially deactivated Florisil, and determination by electron capture gas-liquid chromatography (GLC). Estesen et al. (7) investigated the disappearance of fenvalerate and permethrin from treated cotton and described the details of their determinative procedure.

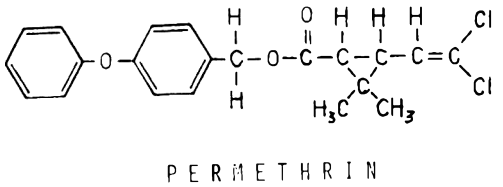
The analytical methods described in the present report are based on existing multi-residue methods for organochlorine insecticides (8, 9); this investigation was undertaken partly with the intent to incorporate pyrethroid residue methodology into the AOAC multi-residue method. The described procedure permits the simultaneous determination of permethrin, cypermethrin, and fenvalerate in vegetable and animal tissue and also allows for concurrent determination of those organochlorine insecticides which have been routinely determined in the past.

METHOD

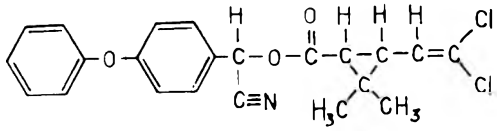
Apparatus and Reagents

(a) *Gas chromatograph.*—Tracor Model 222 with electron capture detector (^{63}Ni source) operated in the constant current mode.

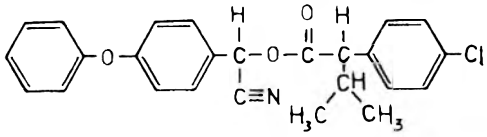
0004-5756/82/6503-0685-05$01.00



PERMETHRIN



CYPERMETHRIN



FENVALERATE

Figure 1. Structural formulas for permethrin, cypermethrin, and fenvalerate.

(b) *GLC columns.*—1.8 m × 2 mm id Pyrex packed with Ultra-Bond® 20M (RFR Corp., Hope, RI); pre-conditioned 48 h at 240°C under nitrogen flow of 20 mL/min.

(c) *Blender.*—Waring, or equivalent, 1 L jar capacity.

(d) *Chromatographic tubes.*—22 mm id × 40 cm with Teflon stopcocks and solvent reservoirs.

(e) *Reference standards.*—98+% cis and trans permethrin, 98+% technical cypermethrin, and 98+% technical fenvalerate (Imperial Chemical Industries Ltd, UK, available through Chipman Inc., Stoney Creek, Ontario, Canada).

(f) *Florisil.*—60-100 mesh (Floridin Co., Pittsburgh, PA). Heat 24 h at 135°C before use and store in sealed container at room temperature.

(g) *Solvents.*—Redistilled acetonitrile, hexane, dichloromethane, and 2,2,4-trimethylpentane (Caledon Laboratories Ltd, Georgetown, Ontario, Canada).

Sample Preparation

Rinse intact vegetable samples lightly with water to remove adhering soil particles and shake dry; where necessary to obtain a practical sample size, remove symmetrical section(s) from original samples; macerate and composite in food chopper. Macerate meat tissue in household-type food processor until homogeneous puree is obtained. Extract milk and egg yolks directly as described in following section.

Extraction

(a) *Vegetable tissue.*—Transfer 50 g subsample of homogenized tissue to blender jar. Add 170 mL acetonitrile and enough water to bring total liquid volume to 250 mL (with allowance for sample moisture content and volume shrinkage between acetonitrile and water). Blend 5 min at high speed and filter 125 mL aliquot (equivalent to 25 g sample).

(b) *Beef muscle and egg yolk.*—Blend 25 g muscle puree or egg yolk 5 min at high speed with 250 mL acetonitrile-water (85 + 15). Let suspension settle and filter ca 125 mL with vacuum, collecting filtrate in graduated cylinder. Place filtrate in freezer at −20°C until water and lipids are frozen, and decant 85 mL supernatant acetonitrile phase (equivalent to 10 g sample aliquot).

(c) *Milk.*—Blend 100 g milk plus 200 mL acetonitrile 5 min at high speed and filter aliquot equivalent to 50 g sample.

Partitioning

Transfer filtered sample aliquots to 1 L separatory funnel, add 100 mL hexane, and shake 30 s. Add 400 mL 2% sodium chloride in water and shake again 1 min. Let phases separate and discard aqueous layer. Rinse hexane extract by shaking gently 30 s with 50 mL water. Drain hexane through 2-3 cm anhydrous Na_2SO_4 and collect in 250 mL boiling flask; rinse separatory funnel with 25 mL hexane and combine with extract by passage through Na_2SO_4. Concentrate to ca 5 mL volume with a rotary vacuum evaporator at 45°C preparatory to column cleanup.

Column Chromatographic Cleanup

Introduce 25 g Florisil into chromatographic column and tap sides of column to produce even packing; top with 1 cm layer of anhydrous Na_2SO_4 and pre-wash column with 50 mL hexane. Quantitatively transfer concentrated hexane extract to column, using 3 successive small rinses (2-3 mL) of hexane; as last of sample enters top of adsorbent, rinse inside of column with additional 5 mL hexane.

Elute sequentially at ca 5 mL/min with (A) 200 mL dichloromethane-hexane (20 + 80) and (B) 200 mL acetonitrile-dichloromethane-hexane (0.35 + 50 + 50), collecting eluates in 300 mL

Received March 17, 1981. Accepted December 8, 1981.

boiling flasks. Evaporate eluate fractions just to dryness with rotary vacuum at 45°C and re-dissolve in measured amounts of trimethylpentane to give analysis solutions with equivalent concentrations of vegetable, meat, and egg at 5 g/mL and milk at 10 g/mL.

Permethrin, cypermethrin, and fenvalerate are contained in eluate fraction B.

Gas-Liquid Chromatography

Measure permethrin, cypermethrin, and fenvalerate by GLC with electron capture detection, using the following operational parameters: column temperature, isothermal at 220°C; nitrogen carrier gas at 100 mL/min; detector temperature, 350°C; injection volume, 5 μL.

Recoveries and Extraction Efficiency

Recoveries were determined at fortification levels ranging from 0.01 to 1.0 ppm with respect to each of permethrin, cypermethrin, and fenvalerate. Duplicate fortifications were made directly onto celery, beef muscle, egg yolk, and milk before the blending operation, and the fortified samples were then carried through the procedure as outlined.

The efficiency of extraction by blending with acetonitrile was determined by comparison with exhaustive methanol-$CHCl_3$ Soxhlet extraction (10). Field-treated samples of celery (4 replicates) were analyzed 3 days following the application of the 3 pyrethroids at rates of 0.08 kg/ha. Egg yolks from laying hens (3 replicates) were analyzed 7 days following the dermal application of permethrin to hens at the rate of 20 mg permethrin per bird.

Results and Discussion

Various methods of extraction and cleanup of the individual pyrethroids are described in the literature; however, because there are close similarities in chemical structure, the use of a common procedure for extraction and cleanup was deemed feasible. The described method is based on current multi-residue technology for organochlorine compounds and is therefore readily compatible with methods now in use for monitoring and screening programs. The use of acetonitrile as an extractant for fatty foods (e.g., meat, egg yolk) is a slight departure from recommended procedure but the property of relatively low fat solubilities in acetonitrile combined with the freeze-out operation results in extracts which are sufficiently free of lipids and which do not overload the Florisil cleanup column.

The column cleanup procedure, originally described by Mills et al. (10), offers superior rejection of lipid co-extractives compared with the elution system of 6% and 15% ethyl ether in hexane. Permethrin, cypermethrin, and fenvalerate elute completely in the second fraction which would also contain, if present in the sample, organochlorines such as dieldrin, endrin, heptachlor epoxide, endosulfan, and methoxychlor. The retention times of the pyrethroids on GLC columns, however, are considerably longer so that no interferences between the analytes occur.

Typical gas chromatograms of celery and egg yolks fortified at levels of 0.1 ppm permethrin and 0.2 ppm cypermethrin and fenvalerate are shown in Figures 2A and 2B. Under the specified GLC conditions, permethrin is almost totally resolved into its 2 pairs of enantiomers with retention times of 3.5 and 4.1 min (2.67 and 3.14 relative to *p,p'*-DDT); cypermethrin, which contains a third asymmetric center, is partially resolved into an envelope of 3 peaks with retention times of 8.0, 8.6, and 9.1 min (6.10, 6.52, and 6.95 relative to *p,p'*-DDT); fenvalerate is partially resolved into its 2 pairs of enantiomers with retention times of 11.5 and 14.2 min (8.76 and 10.8 relative to *p,p'*-DDT). Several GLC liquid phases were investigated including 2% OV-1/3% OV-210, 3% OV-210, 3% OV-225, and Ultra-Bond 20M. The nonpolar OV-1 resulted in peaks which showed little or no isomeric resolution and produced essentially one major composite peak for each pyrethroid; a nonpolar phase could, therefore, be the column of choice if quantitations are made on a summation basis without regard for actual isomeric contents. Best resolution of isomers was obtained with the Ultra-Bond 20M, while columns of intermediate polarities produced correspondingly intermediate resolution of isomers.

Ultra-Bond 20M is prepared commercially by extensive deactivation of a diatomaceous earth followed by coating with Carbowax 20M, treatment at high temperature, and then exhaustive extraction with solvents to produce a bonded support having approximately 0.2% loading of the liquid phase. Columns packed with Ultra-Bond 20M have consistently resulted in higher efficiency and better resolution of individual pyrethroid isomers than do conventionally packed columns.

Previous workers (11, 12) have reported that permethrin and fenvalerate are susceptible to thermal decomposition on GLC columns and that repeated injections with large amounts of the

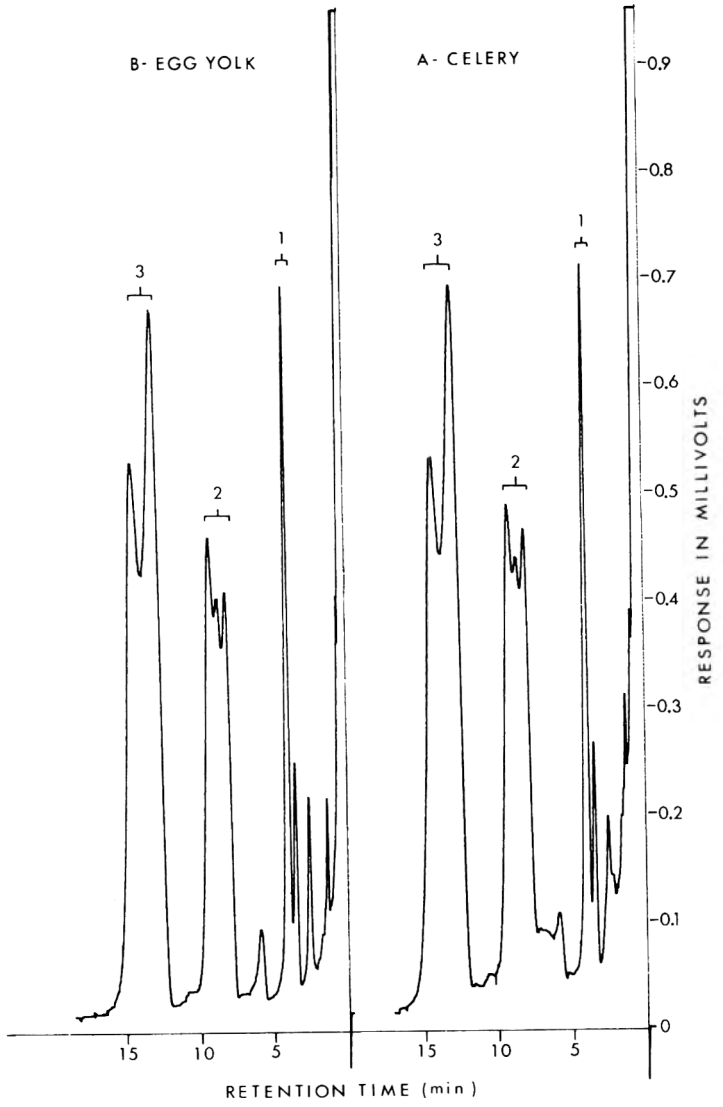


Figure 2. Gas chromatograms of celery and egg yolk fortified with (1) 0.1 ppm permethrin; (2) 0.2 ppm cypermethrin; and (3) 0.2 ppm fenvalerate.

pyrethroids are necessary before peak height reproducibility is acceptable. These difficulties were not observed with the use of Ultra-Bond 20M. Pre-conditioning with heavy injections was not necessary and peak height reproducibility was acceptable. The possibility of on-column decomposition was investigated by using a standard mixture of permethrin, cypermethrin, and fenvalerate in combination with decachlorobiphenyl as an internal standard; peak heights (based on the major peak of each pyrethroid) were monitored over a 4-week period by calculating peak height ratios relative to the internal standard peak (Table 1). The coefficients of

Table 1. GLC comparison of peak height of permethrin, cypermethrin, and fenvalerate vs decachlorobiphenyl as internal standard

	Peak height ratio		
Day	Permethrin	Cypermethrin	Fenvalerate
1	0.87	0.76	0.86
2	0.91	0.77	0.89
4	0.94	0.81	0.91
7	0.90	0.80	0.84
14	0.85	0.75	0.78
21	0.85	0.76	0.79
28	0.89	0.79	0.83
SD	0.033	0.023	0.048
CV, %	3.72	2.96	5.69

Table 2. Average recoveries (2 replicates) of permethrin, cypermethrin, and fenvalerate from celery, beef muscle, egg yolk, and milk

Compound	Added, ppm	Recovery, %			
		Celery	Beef muscle	Egg yolk	Milk
Permethrin	0.01	94.2	82.9	86.8	90.5
	0.10	94.9	89.1	86.0	97.2
	1.0	97.0	89.9	90.9	94.8
Cypermethrin	0.01	93.8	83.4	84.1	91.1
	0.10	102.0	87.6	89.3	91.1
	1.0	97.2	86.9	92.6	94.8
Fenvalerate	0.01	99.2	89.1	85.2	89.0
	0.10	96.1	86.4	81.9	94.4
	1.0	103.4	91.3	88.7	91.3

variation equal to 3.72, 2.96, and 5.69% for permethrin, cypermethrin, and fenvalerate, did not suggest decomposition on the column, nor was there a consistent peak height ratio variation from day 1 to day 28.

With standing current and pulse width adjustments set according to specifications of the instrument and with an attenuation of 4X, peak height responses approximated 3 ng/mV for permethrin, 10 ng/mV for cypermethrin, and 7 ng/mV for fenvalerate. Although these responses are considerably lower than with more highly chlorinated compounds, detection limits of 0.005 ppm are readily attainable for each pyrethroid.

Recovery data at 3 levels of fortification of each of the 3 pyrethroids are shown in Table 2. Values for the 4 substrates represent the average of duplicate determinations. Recoveries for all 3 compounds were good in celery with averages between 94 and 103%. Recoveries from beef muscle, egg yolk, and milk were approximately 10% lower; these lower recoveries are probably associated with the higher fat contents. The comparison of acetonitrile extraction blending vs 12 h Soxhlet extraction with methanol–chloroform (13) is shown in Table 3. No significant differences were evident in the extraction efficiencies of the pyrethroid from field-treated celery. In the case of eggs collected from chickens which had been treated with permethrin, the exhaustive Soxhlet technique appeared to produce slightly higher values for permethrin than were obtained by blending with acetonitrile, but the differences, based on the number of comparisons made, were not statistically significant.

Table 3. Extraction efficiency of acetonitrile blending vs methanol–chloroform Soxhlet extraction, using replicate field-treated samples

Sample	Compound	Residues, ppm	
		Blending	Soxhlet
Celery	permethrin	0.10	0.11
		0.18	0.16
		0.19	0.16
		0.24	0.22
	cypermethrin	0.068	0.077
		0.045	0.041
		0.053	0.049
		0.052	0.067
	fenvalerate	0.16	0.16
		0.26	0.29
		0.16	0.14
		0.22	0.27
Egg yolks	permethrin	0.031	0.034
		0.020	0.023
		0.024	0.029

REFERENCES

(1) Elliott, M. (Ed.) (1977) *Synthetic Pyrethroids*, ACS Symp. Ser. No 42, American Chemical Society, Washington, DC

(2) Williams, I. H. (1976) *Pestic. Sci.* **7,** 336–338

(3) Fujie, G. H., & Fulmer, O. H. (1978) *J. Agric. Food Chem.* **25,** 395–398

(4) Chiba, M. (1978) *J. Environ. Sci. Health* **B13,** 261–268

(5) Chapman, R. A., & Harris, C. R. (1978) *J. Chromatogr.* **166,** 513–518

(6) Lee, Y. W., Westcott, N. D., & Reichle, R. A. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 869–871

(7) Estesen, B. J., Buck, N. A., & Ware, G. W. (1979) *Bull. Environ. Contam. Toxicol.* **22,** 249–257

(8) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **29.011, 29.012**

(9) *Pesticide Analytical Manual* (1980) Food and Drug Administration, Washington, DC, Sec **211.1, 212.1**

(10) Mills, P. A., Bong, B. A., Kamps, L. R., & Burke, J. A. (1972) *J. Assoc. Off. Anal. Chem.* **55,** 39–43

(11) Kikta, E. J., & Shierling, J. P. (1978) *J. Chromatogr.* **150,** 229–232

(12) Mourot, D., Delepine, B., Boisseau, J., & Gayot, G. (1979) *J. Chromatogr.* **168,** 277–279

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

MEAT AND MEAT PRODUCTS

Interaction Between Sample Preparation Techniques and Three Methods of Nitrite Determination

JAY B. FOX, ROBERT C. DOERR, and LEON LAKRITZ
U.S. Department of Agriculture, Science and Education Administration, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118

Meat samples containing nitrite and varying concentrations of ascorbate, cysteine, and sodium chloride were prepared for nitrite analysis. The methods used were the AOAC method of dilution and heating; the addition of mercuric chloride, charcoal, and Carrez reagents at 2 different pH values; and direct analysis of sample supernatants with no treatment (control). The effect of these initial conditions and preparation methods on measured nitrite was determined by using 3 different Griess reagent combinations and chemiluminescent and differential pulse polarographic techniques. Systematic variations were observed in samples treated with mercuric chloride, while the addition of Carrez reagents had little or no effect. Best results were obtained by the AOAC dilution/heating method under alkaline conditions, or by charcoal addition followed by chemiluminescent or colorimetric nitrite determination. Statistical analysis of the nitrite concentrations determined in the several samples showed that these 3 procedures were precise to about 5-6% CV, which was not significantly different from the CV value of 4% determined from replicate analyses.

Sample preparation methods for the determination of nitrite involve a number of processes and reagents designed to clarify the solutions for colorimetric analysis and to improve the yield of nitrite by either cleaving endogenous nitroso compounds (1, 2) or eliminating interferences (3, 4). Common clarifying agents are borate (borax) (1, 2, 5-8), mercuric chloride (1, 2, 6-9), Carrez reagents (1-3, 5-8, 10, 11), iron (Fe^{+3}) (12, 13), and/or aluminum (Al^{+3}) (14-17), all of which are commonly used to precipitate proteins. Mercuric chloride also has been claimed to cleave nitrosothiols, presumed form of the bound nitrite in meats (1, 2). Other procedures included alkalization (1-3, 12-17), dilution of the sample to various levels (1-3, 7, 10, 13, 14, 16-19), and heating (1, 2, 5, 7-11, 13-16, 18, 19).

Evaluations of these methods have always been carried out as entire procedures, without assessments of the effect of the individual steps on either clarification or total nitrite, and seldom as direct comparison with others. Some of the methods involved as many as 4 different reagents and several processing steps, but it is not readily evident that they are all necessary. Furthermore, excessive handling of nitrite samples is not good procedure because nitrite is readily lost through reduction and/or oxidation reactions with endogenous compounds and with oxygen in the air during the preparation procedure. If nitrite is in fact bound to proteins, the use of protein precipitants could lead to a loss of nitrite, depending on the point at which they are used in the preparation procedure.

A systematic comparison of sample preparation methods for nitrite analysis therefore requires a direct comparison of the effect of each reagent or procedure on the amount of measurable nitrite in the sample. In an earlier study of nitrite in frankfurters (20), we used the criteria of maximal nitrite recovery and elimination of turbidity. In that study, the AOAC official first action method (18), which specifies diluting and heating the sample, gave the best results. Recently, we have shown that residual ascorbate has a differential effect on pigment production from different Griess colorimetric reagent combinations (21) and that this effect may be used to determine the effectiveness of sample preparation procedures (4). Using this criterion, it was found that the AOAC method was not as effective as mercuric chloride addition.

We have continued these studies to include other sample preparation methods and nitrite-measuring techniques to determine if there are more effective procedures and/or reagents, and to establish a criterion of accuracy by which to gauge their effectiveness.

Experimental

Reagents and Substrate

All reagents were reagent grade or highest purity obtainable. Darco G-60 activated carbon, tested to be free of nitrite, was used. The meat

Received August 6, 1981. Accepted November 5, 1981.

0004-5756/82/6503-0690-06$01.00

substrate was a 1:2 slurry of pork skeletal muscle and water prepared by first blending an equal weight of pork and 6mM nitrite solution sparged with nitrogen. This slurry was then divided into 4 portions, and to each was added ½ volume of water containing either 0, 9, or 36mM ascorbate or 60mM cysteine. Each portion, containing 2mM ascorbate or 20mM cysteine, was then divided again and each half was made 1M in sodium chloride by the addition of weighed portions of salt. The samples were then heated 1 h at 70°C to react the nitrite with the tissue components and the added reductants. The resulting curd was reblended under nitrogen. The pH of the final slurry was 5.53.

Sample Preparation Techniques

Direct Analysis.—A portion of the slurry was centrifuged and measured volumes of the clear supernate were withdrawn with a syringe pipet for nitrite determination.

Mercuric chloride addition.—Samples were prepared by adding 0.2 mL saturated $HgCl_2$ to 2.0 mL slurry. After 30 min, the slurries were centrifuged and portions of the supernate were withdrawn for nitrite measurement. The precipitates were resuspended and the samples were heated ½ h at 60°C, cooled, and centrifuged. The supernate was again analyzed for nitrite.

For the AOAC preparation, 1 mL slurry was measured by a positive displacement into a 100 mL volumetric flask, diluted to 80 mL, and heated 2 h at 80°C. The flasks were cooled, and the solutions were diluted to 100 mL and filtered through Whatman No. 2 paper which had been washed free of nitrite. The filtrates were analyzed for nitrite.

Carrez reagents addition.—Exactly 0.1 mL Carrez I (0.3M $K_4Fe(CN)_6$) and 0.1 mL Carrez II (1.0M $ZnSO_4$) were added successively to 2.0 mL slurry. After ½ h, the slurries were centrifuged and the supernates were analyzed for nitrite.

Charcoal treatment was performed by diluting 1 mL slurry to 10 mL and adding 0.1 g charcoal. The samples were shaken ½ h and centrifuged, and the supernates were analyzed for nitrite.

Analysis under alkaline conditions.—The effect of alkaline conditions on the mercuric chloride, AOAC, and charcoal preparations was determined by adjusting portions of the slurry to between pH 10.0 and 10.3, which was the pH of most alkaline extractions. The various procedures were performed as before, maintaining the pH as necessary. Because the samples were not buffered, the pH was below 3.0 after addition of the Griess reagents, but within the range of maximal pigment formation (22).

Saturated sodium borate (borax) and Carrez reagents.—Saturated $Na_2B_4O_7$, Carrez I ($K_4Fe(CN)_6$), Carrez II ($ZnSO_4$), and the last 2 together were tested for their effect on the Griess reagents with and without 3mM ascorbate, by adding 0.1 mL of each reagent to 1 mL Griess reagent and 0.1 mL 2mM nitrite, and diluting the solution to 10 mL.

Nitrite Determinations

For Griess colorimetric analysis, a suitable portion of the clear supernate or filtrate was added to 1.0 mL Griess reagents in a 10 mL volumetric flask and diluted to 10 mL. The reagent combinations were sulfanilic acid–*N*-(1-naphthyl)ethylenediamine (SAA), sulfanilamide–1-naphthylamine[1] (SAN), and sulfanilamide–1,7-Cleve's acid (8 amino-2-naphthalene sulfonic acid) (1,7-C). The latter was used to test 1,7-Cleve's acid as a substitute for 1-naphthylamine which has been classified a Class I carcinogen (23). After ½ to 1 h, absorbances were measured in a Cary 14 spectrophotometer.

Nitrite concentrations were also determined by differential pulse polarography (DPP) according to the technique of Chang et al. (24) and by a chemiluminescent technique developed in this laboratory (25). The former method was chosen because it is sensitive to residual ascorbate (24), and the latter because it uses large excesses of ascorbate to generate nitric oxide from nitrite for chemiluminescent detection (CLD) and would therefore be assumed to override the effect of residual ascorbate.

Nitrite content of the various sample preparations was determined by all 3 techniques.

Results

van Eck (26) found that borate slowed the Griess reaction, which, in view of the complex nature of the reaction, might be expected to result in different amounts of pigment formed from different Griess reagent combinations. Preliminary experiments confirmed van Eck's observation on rates, but did not show any reagent combination differences in the final nitrite concentrations. Therefore, we did not investigate the use of borate further, particularly since we had previously found that the reagent had no effect except to increase the pH of the solutions (20).

The addition of Carrez I alone eliminated

[1] 1-Naphthylamine has been classified as a toxic and hazardous substance (23).

Table 1. Effects of NaCl, reductant, sample preparation methods, and Griess reagent on measured nitrite (mM NO_2^-) in pork samples heated 1 h at 70°C ([NO_2^-] = initial 2.0mM)

			Direct		$HgCl_2$			AOAC		Carrez	Charcoal	
NaCl (1)[a]	Reductant (2)	Reagent[b] (3)	Day 1 (4)	Day 2 (5)	H^+[c] (6)	Δ (7)	OH^-[d] (8)	H^+ (9)	OH^- (10)	(11)	H^+ (12)	OH^- (13)
0	0	SAA	1.36	1.35	1.27	1.09	1.25	1.63	1.59	1.38	1.36	1.45
		1,7-C	1.31	1.33	1.16	1.05	1.53	1.58	1.54	1.29	1.43	1.54
		SAN	1.41	1.40	1.24	1.09	1.41	1.62	1.57	1.37	1.47	1.53
	3mM	SAA	1.53	1.47	1.18	0.89	1.09	1.66	1.54	1.47	1.46	1.26
	Asc.	1,7-C	0.50	0.50	1.07	0.80	1.31	0.88	1.24	0.58	1.39	1.36
		SAN	0.86	0.84	1.14	0.79	1.25	1.12	1.38	0.87	1.38	1.36
	12mM	SAA	0.78	0.76	1.02	0.36	0.90	1.01	1.35	0.78	1.06	1.04
	Asc.	1,7-C	0.12	0.12	0.94	0.23	1.08	0.26	0.96	0.14	1.03	1.16
		SAN	0.28	0.28	0.98	0.25	0.98	0.48	1.10	0.27	1.02	1.16
	20mM	SAA	0.86	0.91	1.10	0.61	1.02	1.00	1.35	0.83	1.13	1.17
	Cys.	1,7-C	0.90	0.92	0.99	0.56	1.22	1.06	1.31	0.76	1.12	1.22
		SAN	1.14	1.01	1.07	0.58	1.12	1.13	1.35	0.82	1.13	1.32
1M	0	SAA	1.39	1.47	1.68	1.63	1.21	1.47	1.68	1.25	1.21	1.43
		1,7-C	1.24	1.16	1.45	1.50	1.45	1.40	1.45	1.22	1.19	1.37
		SAN	1.34	1.25	1.50	1.51	1.43	1.44	1.46	1.24	1.16	1.45
	3mM	SAA	0.97	1.00	1.03	0.79	0.68	1.01	1.11	0.88	0.86	0.81
	Asc.	1,7-C	0.44	0.38	0.78	0.72	0.83	0.66	0.91	0.42	0.85	0.83
		SAN	0.58	0.65	0.87	0.73	0.83	0.76	0.91	0.59	0.82	0.83
	12mM	SAA	0.56	0.51	0.83	0.35	0.50	0.67	0.69	0.49	0.67	0.51
	Asc.	1,7-C	0.11	0.10	0.31	0.22	0.58	0.18	0.60	0.09	0.64	0.58
		SAN	0.22	0.19	0.45	0.19	0.58	0.33	0.64	0.18	0.60	0.59
	20mM	SAA	0.93	1.00	1.01	0.59	0.89	0.95	1.12	0.82	0.91	0.90
	Cys.	1,7-C	0.80	0.76	0.91	0.46	1.04	0.89	1.00	0.74	0.85	1.00
		SAN	0.86	0.84	0.92	0.48	0.97	0.91	1.01	0.77	0.85	0.96

[a] Column number.
[b] SAA = reagent combination of sulfanilic acid and *N*-(1-naphthyl)ethylenediamine. 1,7-C = reagent combination of sulfanilamide and 1,7-Cleve's acid. SAN = reagent combination of sulfanilamide and 1-naphthylamine.
[c] Acidic samples, pH 5.53.
[d] Alkaline samples, pH 10.

pigment production from all 3 reagent combinations regardless of the presence of ascorbate or cysteine. Ferrous ions interfere in Griess pigment formation (27) through reaction with the intermediate diazonium ion. This effect was reversed by adding Carrez II which precipitated $Zn_2Fe(CN)_6$, but we suspected that complete reversal of Carrez I inhibition by Carrez II would not occur in meats if sufficient zinc were removed from the system by protein precipitation. Since ferrocyanide is thus contraindicated, we would not have included it in further studies except that it is used in several published methods (3, 5, 8, 11).

The results of the Griess colorimetric analyses of the samples are given in Table 1. The data in columns 4 and 5 are replicates run on 2 different days. The pooled estimate of the standard deviation between replicates was 0.038 ($\bar{X}$ = 0.848, CV = 4.4%). For assessing the results of this study, a given value or average will be considered outside the normal population if it is 4.56 σ (±0.17) or more than the average (28). There was a 30% loss of nitrite in the samples with no added ascorbate or cysteine (first row, Table 1) due to reaction with endogenous compounds, but they had no residual effect on the Griess reaction because all 3 reagents gave the same nitrite concentration. In the samples containing salt, SAA values are higher than are 1,7-C or SAN values due to enhancement of SAA-NED pigment production by chloride (29). With added ascorbate, the effects of enhancement of pigment production with SAA and decreased pigment formation with SAN are observed (4). The interference of ascorbate in pigment production is greater with 1,7-Cleve's acid and is due to the slower coupling rate of the acid with the sulfanilamide diazonium ion as compared with NED or 1-NA (21). Because the interference by ascorbate is due to reduction of the diazonium ion (21), slower coupling reagents produce less pigment in competition with a reductant. Cysteine caused a loss of nitrite, but did not affect the Griess reaction because the difference in measured nitrite values (0.074) is not significantly greater than the expected population variation (±0.038).

Table 2. Effects of NaCl, reductant, and sample preparation methods on nitrite measured (mM NO_2^-) in cured meat slurries by Griess, CLD, and DPP methods

			Preparation method					
NaCl (1)[a]	Reductant (2)	Method[b] (3)	Direct (4)	AOAC (5)	AOAC(OH^-) (6)	Charcoal (7)	$HgCl_2$ (8)	Carrez (9)
0	0	Griess	1.4[illegible]	1.62	1.59	1.46	1.24	1.37
		CLD	1.52	1.52		1.51	1.07	1.16
		DPP	1.00	1.64		1.59	1.06	1.13
	3mM	Griess	0.86	1.12	1.38	1.34	1.14	0.87
	Asc.	CLD	1.44	1.36		1.32	1.00	1.07
		DPP	0.63	1.00		1.30	0.94	0.56
	12mM	Griess	0.28	0.48	1.10	1.11	0.98	0.27
	Asc.	CLD	1.02	1.03		1.19	0.73	0.66
		DPP	0.17	0.40		1.16	0.79	0.23
	20mM	Griess	1.14	1.13	1.17	1.16	1.07	0.82
	Cys.	CLD	1.13	1.15		1.17	0.80	0.44
		DPP	0.58	1.13		1.01	0.64	0.57
1M	0	Griess	1.3[illegible]	1.54	1.46	1.48	1.50	1.38
		CLD	1.48	1.59		1.45	1.28	1.13
		DPP	1.27	1.51		1.45	1.23	1.21
	3mM	Griess	0.58	0.51	0.91	0.93	0.87	0.56
	Asc.	CLD	0.96	0.93		0.90	0.67	0.67
		DPP	0.52	0.16		0.87	0.64	0.52
	12mM	Griess	0.22	0.14	0.65	0.65	0.45	0.20
	Asc.	CLD	0.60	0.65		0.64	0.22	0.35
		DPP	0.02	0.06		0.58	0.30	0.00
	20mM	Griess	0.86	1.09	1.01	1.00	0.92	0.69
	Cys.	CLD	0.96	1.00		0.99	0.44	0.30
		DPP	0.20	0.87		0.87	0.46	0.19

[a] Column number.
[b] CLD = chemiluminescent detection. DPP = differential pulse polarography.

Mercuric Chloride

While the addition of mercuric chloride was largely effective in eliminating the ascorbate effect as judged by the comparable nitrite concentrations measured by the 3 colorimetric reagents, a detailed examination of the data in Tables 1 and 2 raises a question as to the efficacy of the use of $HgCl_2$. Table 1 shows that the amount of measured nitrite for the 3 Griess reagents is in the order SAA > SAN > 1,7-C in the acid-Hg samples (column 6), and 1,7-C > SAN > SAA for the alkaline Hg samples (column 8). This order was common to all samples and is significant at the $P = 0.001$ level. The order in the acidic (H^+) samples is the same as that for residual ascorbate, suggesting that mercuric ion is not totally effective in removing residual ascorbate.

The samples were heated and made alkaline, to determine if the observations of Olsman and van Leeuwen (1) on the release of protein-bound nitrite were applicable to whole meat systems. They found that adding $HgCl_2$ before heating and keeping the pH between 5 and 6 resulted in higher yields of nitrite. Our results show that heating whole meat samples at the lower pH results in overall nitrite loss, while making the samples alkaline results only in reversing the order of pigment yields with the 3 reagent combinations. Although the release of protein-bound nitrite may be a factor in nitrite analysis of cured meats, it is evident that there are other factors involved that are of greater importance. Mercuric ion interfered in the CLD and DPP measurements (Table 2, column 8). The measured nitrite values for these 2 methods were consistently, significantly, and appreciably lower than were the Griess values. The most consistent argument for the lowering is a mercuric ion interference in the nitrosation reaction which is the first step in the sequences involved in all 3 methods of measurement.

Carrez Reagents

The addition of Carrez reagents did not result in any change over direct measurement in the amounts of nitrite measured by the Griess reagents (Table 1) or by DPP (Table 2). They also interfered with the CLD measurement of nitrite in that the Carrez-CLD values were about $\frac{2}{3}$ of the direct measurement values (compare columns 4 and 9, Table 2) with the no added reductant and ascorbate samples, and about one

half with the cysteine samples. To the extent that neither the CLD nor the DPP method requires solution clarification, the finding is superfluous except that it does indicate an interference in nitrosation reactions that probably also takes place in the Griess reaction.

AOAC Method

Use of the AOAC method resulted in an increase in measured nitrite in the meat samples with no reductant but was only partially effective in eliminating ascorbate interference because the residual reductant effect on the different Griess reagents was still observed. Making the samples alkaline almost completely eliminated the residual ascorbate effect (Table 1, column 10) and gave high nitrite values (Table 1, column 10; Table 2, column 6). A pH of 8 has been effective in elimination of ascorbate interference (30, 31) and it is apparent that pH values of 6 or above in the dilute AOAC solutions during heating are effective (32). The effect is attributed to the oxidation of ascorbate at a pH at which nitrite is essentially unreactive (30).

Charcoal Preparation

Of the preparation techniques tested, charcoal gave the most uniform results, both for the 3 Griess reagent combinations (Table 1, columns 12 and 13) and for the 3 different measurement methods (Table 2, column 7). The measured nitrite values for any given reductant level were the same and the pooled σ value for both the H^+ and OH^- charcoal data in Table 1 was 0.0734, indicating no residual ascorbate or cysteine effect. The values so obtained were also the highest for any preparation method tested (Tables 1 and 2).

Chemiluminescent and Polarographic Detection

The nitrite concentrations measured by CLD were the highest and most uniform of any of the measurement methods for the direct, AOAC, and charcoal-treated samples. As previously noted, the addition of $HgCl_2$ or Carrez reagents interfered in the CLD determination. In contrast, the DPP measurements gave low values with added reductants, with an even greater interference by ascorbate in the DPP measurement than was observed in the Griess measurement. Furthermore, there was an interference by sulfhydryl groups in the DPP measurements (direct reading), which was removed by using either the AOAC method or charcoal addition. In view of the sensitivity to interference, the slowness of the procedure, and the inferior results, differential pulse polarography does not recommend itself as a nitrite measurement method.

Discussion

Nitrite Measurement

Because nitrite reacts with the substrate to which it is added, particularly meat products, there can be no absolute value by which to judge the effectiveness of any preparation procedure or nitrite measurement method. Nevertheless, it is only in a system where nitrite has had an opportunity to react that the effectiveness of any procedure can be assessed. Under these circumstances, effectiveness may be gauged by a consensus of those techniques, which by practical experience, on theoretical grounds, or both, give the highest and most consistent yields. Applying these criteria to the present study, the relevant sample preparation and/or nitrite measurement methods are charcoal addition, chemiluminescent detection, and, to a lesser degree, the AOAC method under alkaline conditions. The mean values for the charcoal-treated samples of Table 1 and the CLD, charcoal, and AOAC alkaline method data of Table 2 are listed in Table 3. It is evident that the 2 sets of data are equivalent. The data for 1M NaCl/O reductant do differ significantly, but on examination of the data in Table 1, there appears to be

Table 3. Mean values for nitrite measured (mM NO_2^-) by different sample preparation procedures and nitrite measurement methods

NaCl	Reductant	Charcoal/Griess Table 1 $n = 6$	CLD,[a] Charcoal, AOAC (OH^-), Table 2 $n = 6$
0	0	1.46 ± 0.067	1.52 ± 0.046
0	3mM Asc.	1.37 ± 0.065	1.35 ± 0.054
0	12mM Asc.	1.08 ± 0.065	1.08 = 0.10
0	20mM Cys.	1.18 ± 0.077	1.12 ± 0.065
1M	0	1.30 ± 0.13	1.47 ± 0.017
1M	3mM Asc.	0.83 ± 0.018	0.92 ± 0.038
1M	12mM Asc.	0.60 ± 0.055	0.62 ± 0.033
1M	20mM Cys.	0.91 ± 0.058	0.96 ± 0.059
S_{pooled}		0.073	0.059
CV, %		6.7	5.2

[a] Excluding the $HgCl_2$ and Carrez values.

a systematic error in the acidic charcoal samples. The pooled standard deviations in both sets are less than twice the expected value from replicate analysis (0.038) and we conclude that both sets of data are for the same population, and that the criterion of uniformity is valid. The criterion of maximal yield is also met with respect to ascorbate for the charcoal and AOAC alkaline methods, since they gave the same measured nitrite as did the CLD method. Because the latter generates NO with excess ascorbate, which is a more powerful reductant than sulfhydryl groups, the criterion also applies to the cysteine interference. The results indicate, therefore, that uniform and maximal yield of measured nitrite in meat systems may be obtained colorimetrically with the use of either charcoal or the AOAC alkaline procedure, or by chemiluminescent detection of nitric oxide produced from nitrite by reduction with ascorbic acid. We found that the use of mercuric ion or Carrez reagents is not advisable, either as a preparation procedure or as a step therein.

Removal of Turbidities

All of the referenced methods (1–3, 5–18) filter the samples to remove precipitates generated by the added reagents or procedures. In this study, we used centrifugation to remove precipitates. On occasion, especially in samples containing mercuric chloride, we found that turbidities that were not removable by filtration were readily cleared by centrifugation. As a result, when we tested for turbidity in the samples, we found that it was a negligible factor.

We recommend that the use of charcoal and the AOAC alkaline procedures as sample preparation methods be investigated in a collaborative study, with the objective of replacing the present AOAC method. Such a study should, if possible, include an evaluation of the effectiveness of centrifugation and filtration in removing turbidities from samples before colorimetric analysis. While the use of chemiluminescent determination of nitrite does not appear to be a suitable standard method now because of the time and equipment involved, it may be used as a reference method by which to judge standard methods. Further studies on reagent additions and/or preparation techniques should be strictly confined to the demonstration, by direct comparisons, of the effectiveness, utility, and necessity for the reagent or technique.

REFERENCES

(1) Olsman, W. J., & van Leeuwen, C. M. (1977) *Z. Lebensm Unters. Forsch.* **164,** 239–242
(2) Mirna, A. (1970) Proc. 16th European Meeting of Meat Research Workers **1,** 681–691
(3) Adriaanse, A., & Robbers, J. E. (1969) *J. Sci. Food Agric.* **20,** 321–325
(4) Fox, J. B., Zell, T. E., & Wasserman, A. E. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1397–1402
(5) International Organization for Standardization (1975) ISO 2918–1975(E) Meat and Meat Products, Geneva, Switzerland
(6) Mirna, A., & Schütz, G. (1970) *Jahresbericht der Bundesanstalt fur Fleischforschung* G83-4
(7) Grau, R., & Mirna, A. (1957) *Z. Anal. Chem.* **158,** 182–189
(8) Fudge, R., & Truman, R. W. (1973) *J. Assoc. Public Anal.* **11,** 19–27
(9) Perryman, D. L. (1977) *Lab Prac.* March, 186–187
(10) Sen, N. P., & Donaldson, B. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1389–1394
(11) International Dairy Federation (1978) Standard 84: 1978
(12) Noetzel, O. (1927) *Pharm. Zentralhalle* **68,** 321–323
(13) Nordisk Metodik—Komite for Levnedsmidler (1963) UDC 546.173, Nr49, NMKL, Copenhagen, Denmark
(14) Follett, M. J., & Ratcliff, P. W. (1963) *J. Sci. Food Agric.* **14,** 138–144
(15) Glover, I. T., & Johnson, F. T. (1973) *J. Chem. Educ.* **50,** 426–427
(16) Coppola, E. D., Wickroski, A. F., & Hanna, J. G. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 783–786
(17) Kamm, L., McKeown, G. G., & Smith, D. M. (1965) *J. Assoc. Off. Agric. Chem.* **48,** 892–897
(18) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA secs **24.041-24.042**
(19) Acel, D. (1916) *Z. Unters. Nahr. Genussm. Gebrauchsgegenstaende* **31,** 332–341
(20) Nicholas, R. A., & Fox, J. B., Jr (1973) *J. Assoc. Off. Anal. Chem.* **56,** 922–925
(21) Fox, J. B., Jr (1979) *Anal. Chem.* **51,** 1493–1502
(22) Rider, B. F. with Mellon, M. G. (1946) *Ind. Eng. Chem.* **18,** 96–99
(23) *Code of Feaeral Regulations* (1976) **29,** 1910.1004
(24) Chang, S.-K., Kozeniauskas, R., & Harrington, G. W. (1977) *Anal. Chem.* **49,** 2272–2275
(25) Doerr, R. C., Fox, J. B., Jr, Lakritz, L., & Fiddler, W. (1981) *Anal. Chem.* **56,** 381–384
(26) van Eck, P. N. (1920) *Water* 127
(27) Axelrod, H. D., & Engel, N. A. (1975) *Anal. Chem.* **47,** 922
(28) Steel, R. G. D., & Torrie, J. H. (1960) *Principles and Procedures of Statistics,* McGraw-Hill Book Co., Inc., New York, NY
(29) Hildrum, K. I. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 956–958
(30) Sen, N. P., & McPherson, M. (1978) *J. Food Safety* **1,** 247–255
(31) Sen, N. P., Lee, Y. C., & McPherson, M. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 1186–1188
(32) Malanoski, A. J. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 345–347

Multicomponent Analysis of Meat Products by Infrared Spectrophotometry: Collaborative Study

OLE-CHRISTIAN BJARNØ
N. Foss Electric A/S, 69 Slangerupgade, Hillerød DK-3400, Denmark

Collaborators: W. Arneth; W. Noack; G. Pfeiffer

A new method for rapid multicomponent analysis of meat products based on infrared transmission was tested in a collaborative study with 4 laboratories. A series consisting of 31 samples of different meats, cooked sausage, liver sausage, and fermented sausage was distributed and analyzed at all laboratories for protein, fat, and water by the present method and by well established reference methods. There was no significant difference between the infrared method and reference methods at the 95% confidence level for determination of protein, fat, and water in meat products.

There is a great demand for quality control in the processed meat industry, especially methods for protein determination. Several methods have been reported, but none has been accepted, either because of lack of accuracy or speed or because of the requirement for complicated chemical apparatus.

In the last 10 years, infrared transmission spectroscopy has proved very successful in the analysis of milk for the constituents fat, protein, and lactose. The accuracy for the AOAC official first action method, **16.078-16.097** (1), is about 1.5% relative. This principle has since been extended to meat products (2). The method consists of reacting the meat sample with an alkaline reagent combined with mechanical subdivision to obtain a milk-like emulsion. Protein, fat, and moisture can then be determined by infrared spectrophotometry in much the same way as for milk. An apparatus designed for this purpose consists of spectrophotometer, reactor, reagents, and software for microprocessor connection.

Experimental

The sample series was selected to represent the normal range of constituents found in meat products and comprised 5 cooked sausages, 6 liver sausages, 16 raw materials, and 4 fermented sausages. These samples were also selected because of variation of connective tissue content.

Samples were prepared and distributed from one of the participating laboratories after the following pretreatment: Raw materials were ground in a double knife chopper with plate openings of 2 mm (Möhle Boy FW 70 N) and then frozen. All samples were distributed frozen in cans.

After thawing, samples were re-homogenized in a Moulinex grinder at each of the 4 laboratories. Shortly after remixing, samples were analyzed on the infrared instrument (Super-Scan, N. Foss Electric, 69 Slangerupgade, DK-3400 Hillerød, Denmark) as well as by standard methods for protein, fat, and water. Samples were prepared for IR determination by weighing and transferring 11 g sample to reaction beaker, adding 100 mL reagent, and reacting the mixture 4 min in the reactor. All samples were analyzed in duplicate.

Participating laboratories used different but mostly well established standard methods:

For protein determination, 2 laboratories used Kjeldahl digestion with selenium catalyst, and 2 laboratories used a Kjel-Foss automated method with mercury catalyst (AOAC **24.037**).

Three laboratories analyzed fat: one by soxhlet (AOAC **24.005**), one by Foss-Let (AOAC **24.006**), and one by refractometry with 1-bromo-naphthalene.

Finally, all 4 laboratories determined water: 3 by the seasand/oven method (AOAC **24.003**), and one by microwave oven.

To adjust Kjeldahl results for varying Kjeldahl factors for connective tissue (5.55 for connective tissue protein vs 6.25 for muscle protein), one laboratory analyzed the content of connective tissue protein by measuring the content of hydroxylproline and multiplying by 8.

Results

The collaborative test and evaluation of results was designed according to the *Statistical Manual of the AOAC* (3).

Repeatability (between-sample, within-laboratory variation) was calculated and is presented in Table 1.

Before the calculation of outliers, readout from instruments which were not calibrated had to be adjusted via linear regression vs the best estimate

Received June 30, 1981. Accepted September 22, 1981.

0004-5756/82/6503-0696-05$01.00

Table 1. Repeatability standard deviation, % components

	Fat			Protein			Water		
Lab.	Std	IR	*F*-test	Std	IR	*F*-test	Std	IR	*F*-test
1	0.25	0.18	*	0.47	0.26	*	0.16	0.38	*
2	0.14	0.28	*	0.28	0.20	*	0.12	0.34	*
3	—	0.29		0.25	0.28		—	0.40	
4	0.17	0.22	*	0.25	0.14	*	0.14	0.28	*
Mean	0.19	0.24	*	0.32	0.23	*	0.14	0.35	*

* Significant difference at 95% confidence level.

of the true content of the samples. This best estimate is defined as the mean values of the standard determinations. Using the Dixon test for outliers (3, p. 86), 4 protein determinations by standard methods and 3 by the instrument method were rejected. The mean of the standard determinations was then calculated, and these mean values were corrected for the deviating Kjeldahl factor for connective tissue protein.

Regression analyses were run to calibrate the instrument means vs standard means from all laboratories. The group of fermented sausages did not fit the general regression, and, because it is statistically meaningless to run a separate

Table 2. Percent protein (mean of duplicate determinations, corrected for connective tissue)

		Lab. 1		Lab. 2		Lab. 3		Lab. 4			
Sample	Type	Std	IR	Std	IR	Std	IR	Std	IR	Mean	
I	Cooked sausage	11.16	10.69	11.07	11.06	11.75	—	10.18	10.73	11.04	10.83
II		10.28	9.89	9.81	9.99	10.19	—	10.48	11.10	10.19	10.33
III		15.37	15.49	15.30	15.71	—	15.34	15.17	16.21	15.28	15.69
IV		11.41	11.38	10.58	10.84	10.94	10.51	10.12	10.63	10.76	10.84
V		10.23	9.43	10.14	10.41	10.25	9.79	9.78	10.18	10.10	9.95
L1	Liver sausage	15.95	16.40	15.90	16.06	15.82	16.30	—	15.91	15.89	16.17
L2		15.87	16.63	16.33	16.13	16.20	16.54	15.25	16.14	15.91	16.36
L3		15.95	16.40	16.15	15.99	16.52	16.01	15.27	16.00	15.97	16.10
L4		11.92	12.63	12.02	11.82	12.38	12.54	11.56	12.08	11.97	12.27
L5[a]		11.9	12.29	12.26	12.28	11.90	12.15	11.17	12.19	11.81	12.23
L6		12.34	12.17	11.75	11.52	12.22	12.25	12.68	12.64	12.25	12.15
1	Pork meat	16.75	17.20	16.81	16.91	17.58	16.30	16.55	16.93	16.92	16.84
2[a]		10.24	10.00	10.05	9.75	10.95	9.65	9.37	9.84	10.15	9.81
3		18.44	18.46	18.16	18.49	—	—	18.08	18.67	18.23	18.54
4		12.45	12.40	11.94	12.02	12.80	11.70	11.39	11.97	12.15	12.03
5		17.45	17.66	17.65	16.70	—	17.08	17.40	17.28	17.50	17.18
6[b]		12.27	11.83	12.36	11.55	12.45	11.77	11.69	11.33	12.19	11.62
7[a]		19.89	18.34	19.51	18.94	20.30	18.81	17.72	18.50	19.35	18.65
8		15.79	14.46	15.48	15.25	15.54	15.19	14.53	14.74	15.34	14.91
9	Beef	17.55	18.23	17.95	18.78	18.45	18.33	17.70	18.50	17.91	18.46
10		12.27	12.17	11.92	12.41	12.21	13.02	11.56	12.02	11.99	12.40
11		18.52	19.25	18.67	19.30	19.91	18.91	18.16	19.64	18.82	19.28
12		13.04	13.32	13.08	13.60	13.76	13.80	12.76	13.36	13.16	13.52
13		18.85	18.91	18.92	19.35	19.62	19.55	18.37	18.94	18.94	19.19
14		14.36	14.46	14.58	14.65	15.37	15.00	13.22	14.15	14.38	14.57
15[a]		20.21	19.60	19.46	19.96	21.25	19.97	18.60	19.72	19.88	19.81
16[a]		14.58	14.92	15.03	15.12	15.92	15.10	14.47	15.19	15.0	15.08
Regression equation converting read-out from IR		P = 1.14× P* − 1.64		P = 1.04× P* + 0.64		P = 0.965× P* + 0.58		P = 1.029× P* − 0.04× F* + 0.21			
Correlation coefficient		0.990		0.991		0.984		0.991		0.997[c]	

[a] *F*-test for difference in between-lab. variation significant at 95% confidence level.
[b] *F*-test for difference in mean values significant at 95% confidence level.
[c] This correlation coefficient is corrected for influence from repeatability and between-lab. variation.

Table 3. Key figures from analysis of variance of protein results

Statistic[a]	Protein, % IR	Protein, % Std
Repeatability SD (S_O)	0.23	0.32
Between-laboratory SD (S_L)	0.27	0.53
Between-methods SD (S_M)	0.23	—
Reproducibility SD (S_X)	0.35	0.62
Accuracy SD (S_Y)	0.35	0.53

[a] S_O = repeatability on same sample.
S_L = variation from lab. to lab. on same sample.
S_M = variation of mean standard vs means of all IR measurements.
S_X = statistical sum of S_O and S_L.
S_Y = statistical sum of S_L and S_M.

regression analysis on so few samples, the results on these samples were not included in the rest of the calculations.

Protein results are shown in Table 2, and results from the analysis of variance are shown in Table 3.

Results for fat and water were treated exactly the same. The Dixon test for outliers rejected 4 reference values and 4 IR values for fat. In addition, all results from one sample were rejected because of marked nonhomogeneity. The Dixon test rejected 8 standard water measurements and 6 IR water results.

Discussion

The use of different standard methods probably results in a poorer performance than could be expected with a single reference method; however, this situation more realistically reflects the analytical conditions of actual use. Samples were handled under well defined conditions, but minor variations in homogenization, water loss during re-homogenization, etc., could produce sample-to-sample variation, which is included in the between-laboratory variation.

Repeatability standard deviations (Table 1) are all acceptably low, and significantly lower for

Table 4. Percent fat (mean of duplicate determinations)

Sample	Type	Lab. 1 Std	Lab. 1 IR	Lab. 2 Std	Lab. 2 IR	Lab. 3 Std	Lab. 3 IR	Lab. 4 Std	Lab. 4 IR	Mean	
I	Cooked sausage	27.11	27.23	26.43	27.10	—	26.50	27.2	26.96	26.9	26.94
II		37.24	37.12	37.20	37.60	—	36.83	—	—	37.22	37.18
III		16.47	16.54	15.76	16.60	—	16.49	16.1	16.98	16.1	16.65
IV		—	—	25.19	25.62	—	25.30	25.1	25.74	25.14	25.55
V		24.32	24.22	24.10	25.25	—	24.32	24.2	24.77	24.2	24.64
L1	Liver sausage	34.45	34.51	33.86	33.85	—	33.93	34.7	33.67	34.3	33.99
L2		36.18	35.87	36.02	35.78	—	35.85	34.8	35.28	35.7	35.70
L3[a]		33.69	32.66	32.68	32.59	—	32.65	31.8	—	32.7	32.63
L4		51.04	50.93	50.55	51.05	—	50.09	49.8	51.17	50.5	50.81
L5[a]		51.04	49.6	48.12	—	—	49.30	49.6	49.58	49.6	49.49
1	Pork meat	17.78	17.51	17.26	17.22	—	17.73	17.6	17.43	17.6	17.47
2		49.50	48.31	48.22	48.83	—	48.76	48.1	49.30	48.8	48.80
3		12.15	11.67	11.36	11.57	—	11.96	11.50	12.16	11.70	11.84
4		40.76	40.92	40.66	40.90	—	41.08	40.2	40.66	40.5	40.89
5		22.39	21.89	23.11	22.16	—	22.70	21.4	21.49	22.0	22.06
6		48.43	47.04	48.12	47.57	—	48.46	47.2	48.29	48.0	47.84
7		17.85	17.51	17.50	17.23	—	17.89	17.3	17.45	17.5	17.52
8		35.15	34.9	34.58	34.62	—	34.92	34.2	33.73	34.6	34.54
9[b]	Beef	15.67	15.18	—	15.0	—	15.36	15.6	15.46	15.63	15.25
10		42.27	41.60	40.82	41.87	—	41.81	41.7	42.05	41.6	41.83
11		9.22	8.28	8.58	8.24	—	8.46	8.20	8.84	8.70	8.46
12		32.70	32.18	32.38	32.69	—	31.86	32.1	31.75	32.4	32.12
13		17.56	17.22	17.62	17.03	—	16.98	17.0	17.41	17.4	17.16
14		35.73	35.48	35.43	35.78	—	35.06	35.0	35.37	35.4	35.42
15		12.32	11.97	12.07	12.10	—	11.47	11.9	12.25	12.9	11.95
16[a]		30.46	30.34	30.43	30.66	—	29.89	—	29.87	30.44	30.19
Regression equation converting read-out from IR		F = 0.971X F* + 0.22		F = 0.963X F* − 0.17		F = 0.985X F* + 0.43		F = 0.519X F* + 1.99			
Correlation coefficient		0.9997		0.9992		0.9996		0.9993		0.9999[c]	

[a–c] See footnotes, Table 2.

Table 5. Key figures from analysis of variance of fat results

Statistic	Fat, % IR	Fat, % Std
Repeatability SD	0.24	0.19
Between-laboratory SD	0.31	0.50
Between-method SD	0.19	—
Reproducibility SD	0.39	0.53
Accuracy SD	0.36	0.50

protein by the IR method. The repeatability is significantly lower for water by the reference methods. One laboratory found significantly lower repeatability for fat by the IR method, and 2 laboratories found significantly lower repeatability for fat by the reference method.

The protein results (Tables 2 and 3) are characterized by good agreement between reference values and IR values. Only one sample showed a significant difference between mean values, and the 5 samples with significant differences for between-laboratory variation are all lowest in IR values. The pertinent figures in Table 3 are all acceptably low and at the same level for the IR and reference methods.

The fat results (Tables 4 and 5) are also in good agreement between the IR method and the reference methods. Significant differences for between-laboratory variation were found for 3 samples; 2 were lowest by IR, and one was lowest by the reference method. One of the 27 samples showed a significant difference in the mean value, but this difference is relatively low

Table 6. Percent water (mean of duplicate determinations)

		Lab. 1		Lab. 2		Lab. 3		Lab. 4			
Sample	Type	Std	IR	Std	IR	Std	IR	Std	IR	Mean	
I	Cooked sausage	59.93	59.2	59.77	59.1	—	—	60.37	60.2	60.09	59.5
II		50.36	50.9	50.48	50.0	51.27	—	—	—	50.70	50.45
III		64.74	64.5	64.39	64.0	63.60	64.1	63.81	—	64.14	64.2
IV		—	—	61.57	60.9	60.26	60.2	61.39	61.5	61.07	60.87
V		63.60	63.9	63.04	61.9	—	62.4	62.73	62.6	63.12	62.7
L1[b]	Liver sausage	45.77	46.2	45.89	46.4	45.77	46.4	46.06	—	45.87	46.33
L2[a]		45.26	44.8	45.29	44.4	44.99	44.3	45.44	43.1	45.25	44.2
L3		47.45	48.2	47.24	47.8	47.72	48.0	47.70	46.7	47.53	47.67
L4		34.85	34.7	35.57	34.6	35.51	34.9	35.51	33.1	35.36	34.32
L5		34.35	35.7	36.14	37.3	35.72	36.1	35.81	34.4	35.51	35.9
L6		37.28	38.1	—	36.7	37.77	37.5	—	38.6	37.52	37.7
1	Pork meat	59.07	59.1	59.05	59.3	58.86	59.1	59.64	59.5	59.96	59.2
2		34.83	35.8	35.46	35.9	36.24	35.8	35.80	34.9	35.58	35.6
3		63.40	63.3	63.13	63.10	62.81	63.10	63.39	62.7	63.18	63.0
4		41.35	40.6	41.30	41.1	41.62	41.1	41.71	41.2	41.50	41.0
5		54.10	53.6	53.54	54.6	53.79	54.0	53.34	54.5	53.69	54.1
6		33.50	34.7	34.08	34.8	34.53	34.3	34.03	33.8	34.04	34.4
7		57.05	57.2	56.97	57.2	—	56.8	56.76	57.3	56.93	57.1
8		43.79	44.1	43.95	43.9	—	44.0	43.53	44.8	43.75	44.2
9	Beef	59.70	59.8	59.43	59.6	60.30	59.6	59.12	59.3	59.64	59.60
10		39.98	40.0	40.18	39.9	39.90	39.3	39.83	39.6	39.97	39.7
11		65.54	65.5	65.38	65.7	65.41	65.3	66.17	64.9	65.63	65.4
12		48.15	48.3	48.12	47.4	48.61	48.0	48.26	49.0	48.29	48.2
13		56.94	56.7	56.75	56.9	57.27	56.8	56.66	56.5	56.91	56.7
14		43.63	43.1	43.61	43.2	44.58	43.8	42.30	43.9	43.53	43.5
15		61.58	61.5	61.27	61.1	62.04	61.6	61.33	61.1	61.31	61.3
16		48.29	48.1	48.09	47.8	—	48.6	48.02	48.5	48.13	48.2
Regression equation converting read-out from Samples 1–16		W = −0.145 × W* − 1.059 × F* − 1.893 × P* + 118.9		W = 0.063 × W* − 0.093 × F* − 1.83 × P* + 93.6		W = 0.022 × W* − 0.919 × F* − 0.978 × P* + 92.33		W = 0.122 × W* − 0.399 × F* − 1.287 × P* + 86.46			
Correlation coeff.		0.9931		0.997		0.991		0.990		0.9998[c]	
Regression equation converting read-out from Samples 1–16		W = 0.122 × W* − 1.354 × P* − 0.899 × F* + 87.7		W = 0.09 × W − 0.846 × F* − 0.946 × P* + 82.21		W = 0.203 × W* −0.905 × F* − 0.838 × P* + 87.10		W = −0.509 × F* − 1.515 × P* + 101.09			
Correlation coeff.		0.9995		0.9990		0.9995		0.9988			

[a–c] See footnotes, Table 2.

Table 7. Key figures from analysis of variance of water results

Statistic	Water, %	
	IR	Std
Repeatability SD	0.35	0.14
Between-laboratory SD	0.48	0.40
Between-method SD	0.23	—
Reproducibility SD	0.59	0.42
Accuracy SD	0.53	0.40

(0.38%). The mean value of between-laboratory variation (0.50%) seems to be a little higher than normally expected in this kind of experiment, possibly due to different reference methods, nonhomogeneity of sample, etc., as previously mentioned.

Water results are found by multiple regression with the fat, protein, and water readouts from the IR instruments, a method which is necessary to achieve sufficient accuracy. Unlike fat and protein determinations, raw materials and the finished products should have separate regression equations. Thus, the performance of the IR measurements is reasonable although not totally satisfactory. One sample showed a significant difference for the mean value, as well as for between-laboratory variation (Tables 6 and 7).

The number of outliers is at the same level (about 3%) for the reference methods and for the IR methods on all parameters. Common finished products as well as different raw materials fit the same regression line for fat and protein, but one product group—fermented sausage—correlates with an individual equation.

Conclusion

This experiment, which evaluated the performance of the IR method based on 1500 measurements on 108 samples, shows that common finished products as well as different raw materials fit the same regression line. There was generally no significant difference between the IR method and well established reference methods for protein, fat, and water.

Due to a general lack of carbohydrates in the selected samples, this study did not verify performance for this parameter.

Acknowledgments

The following collaborators participated in this study:

W. Arneth, Bundesanstalt für Fleischforschung, Institut für Chemie und Physik, E.C.-Baumann-Strasse 20, D-8650 Kulmbach, GFR

W. Noack, Fleischwarenbetrieb Oldenburg, Industriestrasse 1, D-2900 Oldenburg. GFR

G. Pfeiffer, Institut für Forschung und Entwicklung der Hertha KG, Daimlerstrasse 2, D-8060 Dachau, GFR

References

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

(2) Bjarnø, O.-C. (1981) *J. Assoc. Off. Anal. Chem.* (1981) **64**, 1392–1396

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

DRUGS IN FEEDS

Rapid Screening Test for Sulfamethazine in Swine Feeds

DANIEL P. SCHWARTZ
U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118

A simple, 10-min qualitative screening test for sulfamethazine (SM) in swine feeds is detailed. The method, which can be run in the field, uses 2 plastic tubes arranged piggyback style. The upper tube contains, from top to bottom, the feed sample (about 1 g), partially deactivated alumina, and an anion exchange resin buffered at pH 5.7. The bottom tube contains a small bed of anion exchanger buffered at pH 7.9, which traps the SM. After percolation of solvent through the system, the SM, if present, is eluted from the pH 7.9 resin and is reacted with Bratton-Marshall reagents to give a pink-to-lavender color. Feeds containing ≥0.15 ppm can be detected. A simple, additional dye concentration step allows for detection of 0.02 ppm, if desired. Only amphoteric primary aromatic amino-containing compounds with a pKa close to that of SM can theoretically interfere. Preparation of permanent color solutions using cobalt and copper acetates in glacial acetic acid is described for the optional establishment of the minimum concentration of SM in the feed. The method offers a simple way to detect some cross-contaminated withdrawal feeds containing >2 ppm SM, which can lead to violative (≥0.1 ppm) residues in swine liver.

The increased supplementation of animal feeds with growth-promoting and disease-allaying drugs has created a parallel demand for adequate methods to quantitate the drugs both in the feed and in the tissue or fluids of consuming animals (1). When large numbers of samples are to be analyzed, a great deal of time, effort, and expense can be saved if rapid screening methods are available, especially those that give low numbers of false positive tests. In this initial effort, we report such a method, namely, a screen for sulfamethazine, by far the major sulfa drug added to swine feeds (2, 3). The method has been specifically designed so that it can be run in the field and in the laboratory with equal facility, using simple, disposable equipment. The highly sensitive Bratton-Marshall (B-M) color test forms the basis for the detection method. The well known nonspecificity of the reaction due to the presence of B-M-positive compounds in feeds has been circumvented by using small, buffered columns of anion exchange resins. Feed samples containing ≥0.15 ppm sulfamethazine can be detected in about 10 min and a simple, optional step allows detection of feeds containing 0.02 ppm sulfamethazine.

There are no screening tests for SM in swine feeds that can be readily used in both the laboratory and the field. However, a rapid screening test designed primarily for sulfadimethoxine in poultry feeds is available, which uses the nonspecific reagent *p*-dimethylaminocinnamaldehyde (4).

METHOD

Apparatus and Reagents

All reagents were stored at room temperature without precaution to exclude light. Deionized or distilled water was used throughout the study.

(**a**) *Sodium nitrite.*—0.12% in water.

(**b**) *Ammonium sulfamate.*—0.8% in water.

(**c**) *N-1-(Naphthyl)ethylenediamine dihydrochloride (NED).*—(Sigma Chemical Co., St. Louis, MO 63178) 0.8% in water containing 0.1% ethylenediaminetetraacetic acid (EDTA).

Solutions (**a**), (**b**), and (**c**) were stored in, and dispensed from, drop dispenser bottles (Nalge 2411 Series, A. H. Thomas Co., Philadelphia, PA 19105). Solutions were usable for 2 months.

(**d**) *Potassium dihydrogen phosphate.*—0.2M. Dissolve 27.8 g KH_2PO_4 in 1 L water.

(**e**) *Dibasic sodium phosphate.*—0.2M. Dissolve 71.1 g $Na_2HPO_4 \cdot 12\ H_2O$ in 1 L water.

(**f**) *pH 5.7 buffer.*—Using pH meter, add (**e**) to 50 mL of (**d**) until pH = 5.7 ± 0.05 pH unit is obtained. If no pH meter is available, add 4.5 mL of (**e**) to 50 mL of (**d**).

(**g**) *pH 7.9 buffer.*—Using pH meter, add (**d**) to 50 mL of (**e**) until pH 7.9–7.95 is obtained. If no pH meter is available, add 4.0 mL of (**d**) to 50 mL of (**e**).

(**h**) *Acidic alumina.*—Add 10 mL pH 5.7 buffer

Received August 26, 1981. Accepted October 14, 1981.
Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

0004-5756/82/6503-0701-05$01.00

to 90 g alumina (Fisher, No. A-948) in screw-cap bottle and shake until all lumps are broken. Do not substitute any other alumina.

(i) *Resin.*—Dowex 1X2, 100–200 mesh (Sigma).

(j) *Transfer pipets.*—Polyethylene. Pasteur-pette, bulb-type (Centaur Chemical Co., 180 Harvard Ave, Stamford, CT 06902).

(k) *Pipet tips.*—5 mL (Rainin Instrument Co., Mack Rd, Woburn, MA 01801).

Optional Apparatus and Reagents

(a) *Dowex 50-cellulose powder.*—Weigh equal amounts of Dowex 50X4 (100–200 mesh) (Sigma) and cellulose powder CF-11 (Whatman, Inc., Clifton, NJ 07014) and grind lightly (mortar and pestle) until light tan.

(b) *Cobaltous acetate solution.*—2%. Weigh 2 g ± 5 mg $Co(OAc)_2$ and dissolve in 80–90 mL glacial acetic acid in 100 mL volumetric flask. When completely dissolved, dilute to volume with glacial acetic acid and mix.

(c) *Cupric acetate solution.*—0.015%. Weigh 150 mg $Cu(OAc)_2 \cdot H_2O$ and dissolve in 100 mL glacial acetic acid as above.

(d) *Alumina scoop.*—To obtain ca 1 g alumina. Laboratory-made as follows: Mark Pasteur-pette 1 and 3 cm from bulb end. Insert point of single-edge razor blade at one seam and cut along mark to other seam. Make similar cut at other mark. With scissors, cut out a "window" along the seams from end of one razor cut to other. Cut off tip of pipet to facilitate transfer of alumina. After scooping up alumina, hold scoop vertically and tap at top with index finger to dislodge excess alumina. Then transfer alumina by letting it slide down barrel.

(e) *Feed scoop.*—For transferring ca 1 g feed samples. Make as described above except make first cut 1.5 cm from end of bulb.

(f) *Pressure bulb.*—Convenient apparatus to apply air pressure (A. H. Thomas, No. 1957-K10).

Procedure

Preparation of pH 5.7 and 7.9 resins.—Place 10 g (as received) Dowex 1X2 resin in 30 mL coarse sintered glass funnel. Wet resin with water to settle and let drain. Add 50 mL pH 5.7 phosphate buffer and let solvent percolate by gravity flow. After all of buffer has entered bed, force excess out by pressure or vacuum application and wash resin with ca 50 mL water until effluent emerges neutral (pH paper). Force out excess water and transfer the equilibrated resin to suitable container such as 60 mL narrow-mouth polyethylene bottle with screw cap. Add 50 mL 95% ethanol. Prepare pH 7.9 resin in similar fashion using pH 7.9 phosphate buffer.

Preparation of pH 7.9 tube.—Cut 3 cm off tip and 0.5–1.0 cm off top of bulb portion of transfer pipet. Insert small wad of fine glass wool into bottom of barrel portion. Transfer 1 mL of a magnetically stirred suspension of pH 7.9 resin to tube, using pipet with relatively large tip opening. Let excess alcohol drain and wash down pipet walls with alcohol. Place small wad of glass wool on top of resin bed, but do not tamp. If no magnetic stirrer is available, suspend resin by shaking and pipet immediately.

Preparation of pH 5.7-alumina-feed tube.—Plug bottom of a 5 mL pipet tip with small wad of glass wool and transfer 1 mL of a magnetically stirred suspension of pH 5.7 resin and let alcohol drain. Place 1 g alumina on top of resin bed. Then add 1 g of feed sample. Feed sample should be homogeneous, representative of the lot or batch, and preferably ca 20–200 mesh. (An inexpensive (<$20) coffee mill (Waring Products, Route 44, New Hartford, CT 06057, also available in some retail stores) is used in our laboratory to grind feed 10 s.)

Extraction of sulfamethazine.—Place tip of prepared pH 5.7-alumina-feed tube piggyback in pH 7.9 tube (Figure 1) and add 5 mL ethyl acetate–methanol–water (7 + 2.7 + 0.3). After solvent has completely passed through beds in both tubes, remove and discard upper tube; then pipet (or squirt to a premark) ca 1 mL 95% ethanol into bottom tube. Let ethanol drain and add 2 mL water and let drain. Place 1.9 mL (½ dram) lipless (shell) vial under tube and add 0.8 mL 3.5N aqueous HCl containing 0.01% Triton X-100 (Sigma) to elute sulfamethazine from pH 7.9 resin. Add 1 drop of reagents (a), (b), and (c) in sequence to the effluent, shaking vial 10–15 s between additions. Pink-to-lavender color indicates presence of sulfamethazine. Feeds containing ≥0.15 ppm will give positive response. If color is not apparent in vial, lower limit of detectability can be increased ca 7.5 times (to 0.02 ppm) by following procedure: Cut 3 cm off tip of a disposable glass Pasteur pipet (5¾ in. long) and dab new tip into Dowex 50-cellulose powder mixture until short (0.2–0.4 cm) bed of powder is retained in tip. Wipe excess powder from outside and push tip into a tightly compacted bed of fine glass wool contained in a vial. Gently twist pipet until small plug of glass wool has been retained to support bed. Tap tip on solid surface to settle powder and give an even bed surface. Add 0.5 mL methanol to reaction vial,

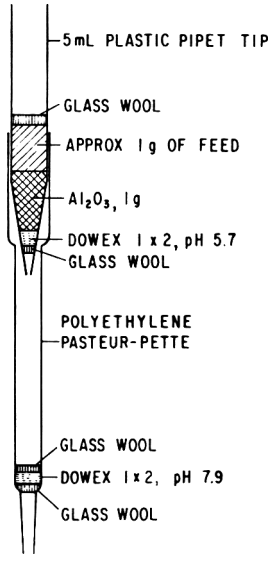


Figure 1. Setup of tubes for sulfamethazine screening test.

stir, and transfer to Pasteur pipet. Presence of narrow lavender band forming at top of bed as part or all of solution passes over bed indicates presence of sulfamethazine.

Preparation of Permanent Color Standards

Accurately pipet 1.7 parts cobaltous acetate solution, 0.5 part cupric acetate solution, and 2.8 parts glacial acetic acid, and mix. This solution will then contain color intensity equivalent to color obtained when running Bratton-Marshall (B-M) reaction on 2 μg sulfamethazine/mL reagents used in this study (3.5N HCl and 1 drop each of reagents (a), (b), and (c)). This would be equivalent to 2 ppm in feed, assuming quantitative recovery. To prepare solutions with colors equivalent to lower concentrations of (B-M) dye, dilute colored solution with glacial acetic acid, e.g., for 1 μg/mL dilute 1:1, for 0.25 μg/mL dilute 0.25:1.75, etc. To ensure against subtle changes in color of standards due to evaporation of acetic acid, seal 1 mL volumes in 2 mL glass ampules (Ace Scientific Supply Co., PO Box 127, Linden, NJ 07036, No. 10-1248-17).

Results and Discussion

Approximately 100 different feed samples containing various amounts of sulfamethazine were screened using the procedure. At least 3 analyses were made on each feed because of modifications made to the original procedure. The feed samples were primarily corn- or alfalfa-based or were mixtures of the two. A few samples were reddish in color, were heavy, and apparently contained $CaCO_3$ because they released gas when acidified. The majority of samples contained less than 0.5 ppm as determined by specific quantitative and semiquantitative chromatographic methods in other laboratories that routinely conduct analyses, or which were spiked with levels of sulfamethazine ranging from 0.05 to 0.5 ppm in this and in other laboratories. Samples that were reported to contain no sulfa drug were always negative in our screening procedure or showed just a trace (using the micro Dowex-cellulose column) of sulfa drug. A clear, colorless effluent was always obtained from the pH 7.9 resin and the resultant color with the B-M reagents was always true, thereby reducing doubt as to the positive nature of the sample Samples spiked with varying amounts yielded color intensities in the procedure that could be ranked correctly as to relative concentration.

The recovery of sulfamethazine from the feed sample is not quantitative and can vary from one type of feed to another and also with the concentration. The efficiency of the extraction procedure appears to be the major drawback in making the procedure quantitative. Therefore, the B-M color that is obtained can not be completely correlated with the actual amount of sulfamethazine in the sample. If one wishes to match the color obtained with standards prepared each day, or, more conveniently, with the permanent color standards, then an approximate *minimum* concentration of sulfamethazine in the feed can be established. Using the procedure, cross-contaminated withdrawal feeds containing >2 ppm, which can lead to >0.1 ppm violative residue in swine liver (5), can be readily ascertained. The eye cannot differentiate the intensity of the dye produced by the B-M reaction when the concentration of sulfamethazine is above about 2 μg/mL, so dilution is necessary in these instances.

Single samples of feed can be screened in about 10 min unless the feed sample has been too finely ground, in which case it will take longer. When properly set up (with vial racks and tube supports) over 100 samples can be run easily by one person in an 8 h day. Moreover, this can be accomplished in about 1 sq. ft of space. If many samples are to be run simultaneously, speed of analysis will not be an important factor.

Some sensitivity has been sacrificed to reduce analysis time. If the pH 7.9 column is made to run slower, quantitative exchange of sulfamethazine will occur (as opposed to about 90%),

resulting in about 10% increase in sensitivity. Sensitivity can also be increased if the resin is eluted with a smaller volume of acid, e.g., 0.4 mL instead of 0.8 mL, thereby doubling the color intensity and increasing the lower limit of detection from 0.15 ppm to between 0.07 and 0.08 ppm in the vial. Both of these parameters can be accomplished by using 0.5 mL of the pH 7.9 resin suspension instead of 1 mL, and making the column in the narrower diameter tip portion of the Pasteur-pette instead of in the barrel portion. This will reduce the flow rate from under 10 min to between 20 and 40 min, depending on how far down in the tapered tip the resin bed is located. This option is available to the analyst depending on assay needs; the micro Dowex 50-cellulose column described earlier may be used to detect even lower concentrations.

Specificity of the Method

Theoretically, only amphoteric compounds which contain a primary amino functional group on a benzene ring can interfere in the analysis and give a false positive reaction. Unlike the procedure that Tishler et al. (6) designed, in part, to limit interferences to ampholytes by using strong acid and strong base extractions, in our procedure a narrow pH range (5.7–7.9) is used for the exchange of sulfamethazine onto the resin. Thus only qualifying ampholytes with acid dissociation constants very close to sulfamethazine can possibly interfere. This range was established by equilibrating the resin against buffers of varying pH values and determining the amount of SM exchanged onto the resin. No exchange occurred below pH 5.7, and the amount exchanged increased to a maximum (100%) at or above pH 7.9. Although a number of sulfa drugs have a pKa very close to that of sulfamethazine (7, 8) and will exchange in this range, others, such as sulfathiazole, will exchange at pH 5.7 or below and will be removed in the upper trap column. Sulfamethazine and sulfathiazole, the major sulfa drugs used in swine feeds, were put through the entire system in the absence of feed at the 1–2 μg level and were recovered at 91 and 0%, respectively. Some naturally occurring ampholytes which qualify as potential false positive reactors in the analysis, e.g., *p*-aminobenzoic acid, anthranilic acid (*o*-aminobenzoic acid), and kynurenine (3-anthraniloylalanine) as well as sulfanilic acid, a possible breakdown product of sulfa drugs, will also be removed in the upper trap column. Alumina (deactivated to a point where sulfamethazine will not adsorb) is used primarily to adsorb some interfering plant pigments. It also has a strong affinity for relatively strong carboxylic (and sulfonic) acids, thereby further reducing the number of compounds qualifying as potential false positive B-M reactors.

Triton X-100 (Sigma) was incorporated in the 3.5N HCl after we noted that some feed samples contained lipids (presumably water-insoluble acids) that coated the resin and made elution of SM slow and difficult. Triton X-100, a non-ionic wetting agent which facilitates the wetting and subsequent exchange of Cl^- for SM anions on the resin, has no effect on the diazotization of SM and subsequent coupling with NED.

Permanent Color Standards

There is an obvious need, especially in field screening, for permanent color standards to simulate the colors obtained in the B-M reaction and to give the analyst at least an approximation of the minimum concentration of SM present in a feed sample. These permanent color standards should find greater utility, however, in more quantitative procedures.

Attempts to prepare permanent color standards using the B-M reaction product with sulfamethazine were unsuccessful due to fading of the dye. This occurred even when the dye solution was sealed in an inert atmosphere and in the presence or absence of ascorbic and/or isoascorbic acid as antioxidants and with the solutions stored in the dark at 4°C. The colors could be simulated very well by using some organic dyes such as alkaline solutions of phenol red, but these also faded rapidly even under inert conditions. The use of cobaltous and cupric acetates in glacial acetic acid in the recommended ratios gives colors that are virtually indistinguishable to the eye from the corresponding B-M dye. These colors are unaffected by light, time, and oxygen, and, barring losses of acetic acid from evaporation, should remain unchanged for prolonged periods. Glacial acetic acid was the only solvent found for the salts in which the desired color could be obtained; water, methanol, and 95% ethanol were unsatisfactory.

Stabilization of NED

One of the anticipated problems in using the screening procedure in the field was the known instability of NED solutions. Thus, analysts with no access to an analytical balance would have to resort to a more inaccurate way to prepare fresh NED solutions. The stability of NED solutions prepared with water from various sources was studied. We found that tap water, or distilled

water that had been in contact with metal, gave NED solutions that began to darken within hours after preparation. Solutions of NED in deionized water, however, did not begin to darken for several days. The addition of EDTA to water from any source stabilized the NED (presumably against metal-catalyzed oxidation) by delaying the onset of darkening for 3–4 weeks and subsequent deterioration of the NED which is manifested by precipitation. Concentrations of EDTA (from 0.05 to 0.20%) were equally as effective in stabilizing NED in the 3 types of water studied. Even after 2 months, the addition of EDTA-stabilized NED to diazotized sulfamethazine gave the same color yield as did freshly prepared NED in deionized water.

Acknowledgments

The authors thank R. Bevill and his associates at the University of Illinois, College of Veterinary Medicine, for the majority of the corn-based samples together with their analytical results; W. Steller (American Cyanamid), Carolyn Andres (Food and Drug Administration (FDA), Denver), and G. Salmon (FDA, Kansas City) for kindly supplying feed samples of known sulfamethazine concentration; and W. Fiddler, of this laboratory, for patience and encouragement.

References

(1) Horwitz, W. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 104–130
(2) Teague, H. S., Frobish, L. T., & Gerrits, R. J. (1981) *Feedstuffs* **53,** 1, 42
(3) Bevill, R. F. (1980) USDA-FDA-Swine Industry Task Force Report on Sulfonamide Residue Self-Help Program, Washington, DC, January 1980
(4) "Rofenaid 40 Quick Test—For Rapid Estimation of Sulfadimethoxine Levels in Withdrawal Feeds" (1979) RCD 5671/1279, Hoffmann-LaRoche, Inc., Nutley, NJ
(5) Lloyd, W. E., Jenny, A. L., Cox, D.F., & Rottinghaus, G. E. (1981) *Am. J. Vet. Res.* **42,** 339–343
(6) Tishler, F., Sutter, J. L., Bathish, J. N., & Hagman, H. E. (1968) *J. Agric. Food Chem.* **16,** 50–53
(7) Bell, P. H., & Roblin, R. O., Jr (1942) *J. Am. Chem. Soc.* **64,** 2905–2917
(8) Debendetti, P. G., & Rastelli, A. (1978) *J. Med. Chem.* **21,** 1326–1327

High Performance Liquid Chromatographic Determination of Clocapramine in Feed and Plasma

AZIZ GEAHCHAN, PAUL CHAMBON, and PIERRE GENOUX
Laboratoire de Toxicologie, Faculté de Pharmacie, 8, avenue Rockefeller, 69373 Lyon, France

A high performance liquid chromatographic method is described for the determination of clocapramine in animal feed and plasma. Samples are made alkaline and then extracted with chloroform containing opipramol as internal standard. For plasma samples, the organic phase is evaporated to dryness under a stream of nitrogen, and the residue is dissolved in dichloromethane-methanol. Extracts are chromatographed on silica gel with dichloromethane-methanol-ammonia (100 + 10 + 0.25) as eluant, and quantitated using an internal standard. Within-day precision for plasma extracts (n = 15) was 3.39, 5.7, and 4.13% at 5, 10, and 15 mg clocapramine/L plasma, respectively, and day-to-day precision was 4.6, 6.8, and 4.4% at the same levels. The detection limit was 0.5 mg/L. Recovery from feed over the concentration range 2-6 g/kg was >96%.

Clocapramine, 1′-(3-(3-chloro-10,11-dihydro-5*H*-dibenz(b,f)azepin-5-yl)propyl)(1,4′-bipiperidine)-4-carboxamide (substance 1, Figure 1), is a new neuroleptic agent which shows imipramine- and chlorpromazine-like activities that distinguish it from other neuroleptic drugs. To accurately estimate clocapramine concentration in animal feed and to determine low blood levels after oral administration of low therapeutic doses, a rapid, accurate, sensitive method is needed.

Various methods have been reported for analysis and separation of tricyclic psychosedative drugs, using ultraviolet spectrophotometry (1), fluorometry (2), thin layer chromatography (3, 4), and gas-liquid chromatography (5, 8). These methods are not sensitive enough or require a lengthy cleanup to eliminate interference from endogenous compounds.

High performance liquid chromatography has been used for separating a number of commercially available tricyclic antidepressants (9–11) and tranquilizers. Several separation systems have been reported using adsorption chromatography (12–15), reverse phase chromatography (16, 17), and ion-pair liquid chromatography (18, 19). These methods are rapid and reproducible.

This paper reports the development of a new method for determining clocapramine in feed and in human and dog plasma by using high performance liquid chromatography on silica gel, with internal standardization. The method is simple and sensitive, and is capable of estimating low levels by a rapid extraction procedure.

Received July 9, 1981. Accepted November 6, 1981.

METHOD

Apparatus

Equipment specified is not restrictive; other suitable equipment may be used.

(a) *Liquid chromatograph.*—Chromatem Model M 3 (Touzart & Matignon, Vitry sur Seine, France) coupled to 254 nm ultraviolet absorbance detector (Altex, Berkeley, CA 94710) equipped with 8 μL flow cell. High pressure sample injection valve fitted with 20 μL sample loop injector (Rheodyne, Inc., Berkeley, CA 94710).

(b) *HPLC recorder.*—Sefram (Sefram, Paris, France), with setting at 10 mV and chart speed of 0.5 cm/min.

(c) *Integrator.*—Hewlett-Packard Model 3380 A (Hewlett-Packard Co., Palo Alto, CA 94304) with connections for liquid chromatograph.

(d) *Column.*—150 × 4.6 mm id stainless steel, packed by slurry technique with 5 μm LiChrosorb Si-60 (Merck, Darmstadt, GFR).

Reagents

(a) *Solvents.*—Reagent grade $CHCl_3$, methanol, and ammonia. For HPLC elution solvent, use chromatographic grade dichloromethane (Merck, or equivalent).

(b) *Standards.*—(1) *Animal feed.*—Standard samples were prepared by personnel who normally prepare diets for dosing. To 50 g feed (B04, UAR, Villemoisson, France), add 100, 200, and 300 mg clocapramine as hydrochloride, respectively, to give 2, 4, and 6 g/kg. Carefully homogenize standard feed preparations, blend by hand in mortar in one or several operations. (2) *Plasma.*—Prepare methanolic stock solutions by dissolving 25, 50, and 75 mg clocapramine as hydrochloride in 20 mL methanol to give stock solutions containing 1.25, 2.5, and 2.75 g/L, respectively. Quantitatively transfer 1 mL aliquot of each stock solution to 25 mL volumetric flasks

0004-5756/82/6503-0706-05$01.00

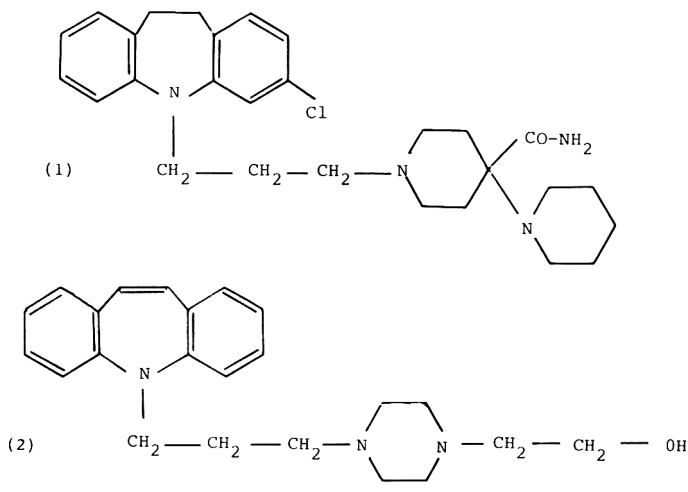


Figure 1. Structures of (1) clocapramine and (2) opipramol.

and dilute to volume with distilled water. Aqueous solutions contain 50, 100, and 150 μg clocapramine/mL. Quantitatively transfer 2 mL of each aqueous solution to 20 mL volumetric flasks and dilute to volume with blank plasma obtained from healthy, untreated animals. Plasma standards contain 5, 10, and 15 μg clocapramine as hydrochloride/mL.

(c) *Internal standard solutions.*—(*1*) *Animal feed extraction solution.*—Accurately weigh 400 mg opipramol (substance 2, Figure 1) as hydrochloride and quantitatively transfer to glass-stopper 100 mL volumetric flask. Add 5 mL 1N NaOH and 40 mL $CHCl_3$, and shake mixture 10 min. Transfer to 125 mL separatory funnel. Drain $CHCl_3$ layer (lower) into 50 mL Erlenmeyer flask containing 5 g Na_2SO_4. Quantitatively transfer 10 mL aliquot of $CHCl_3$ phase containing opipramol to 100 mL volumetric flask and dilute to volume with $CHCl_3$ to give internal standard stock solution containing 1 mg opipramol/mL. This organic phase was used without further dilution to extract animal feed samples. (*2*) *Plasma extraction solution.*—Quantitatively transfer 1 mL aliquot of internal standard stock solution to 200 mL volumetric flask and dilute to volume with $CHCl_3$ to give solution containing 0.5 μg opipramol/mL.

Sample Extraction

Animal feed.—Use this procedure for concentrations from 0.5 to 6 g/kg. Accurately weigh 2 g feed (or animal feed standards) and quantitatively transfer to 100 mL glass-stopper volumetric flask. Add 5 mL 1N NaOH. With magnetic stirring bar, stir mixture 5 min at room temperature. Add 20 mL internal standard stock solution and extract 10 min on mechanical shaker. After 2 phases separate, inject 20 μL of $CHCl_3$ extract into liquid chromatograph.

Plasma.—To 1 mL plasma sample (or plasma standards) in 10 mL glass-stopper centrifuge tube, add 0.2 mL 1N NaOH and 5 mL plasma extraction solution. Shake and centrifuge 10 min at 2200× g. Use Pasteur pipet to transfer organic layer to dry, clean 5 mL test tube and evaporate to dryness in 50°C water bath under stream of nitrogen. Dissolve residue in 0.5 mL mobile phase mixture.

High Performance Liquid Chromatography

Perform HPLC analysis under following conditions: flow rate 0.9 mL/min; pressure 30 bars; ambient temperature; recorder chart speed 0.5 cm/min; mobile phase dichloromethane–methanol–ammonia (100 + 10 + 0.25). Filter mobile phase under vacuum through 0.45 μm membrane and degas by sonication for 15 min under vacuum. Monitor column effluent at 254 nm with sensitivity of 0.64 AUFS for feed extracts and 0.04 AUFS for plasma extracts. Under these conditions, retention times for clocapramine and opipramol were 3.5 and 4.7 min, respectively. Capacity factors, K', of the 2 substances were 1.02 and 1.70, respectively.

Plasma and feed concentrations were determined from standard curves established by plotting clocapramine:opipramol response ratios against clocapramine concentrations. The method was validated for peak height and area ratios.

Table 1. Linear regression of clocapramine:opipramol response ratio vs clocapramine concentrations (plasma samples)

Clocapramine concn, mg/L	Area ratio	SD (n = 15)	RSD, %
5	0.361	0.017	4.6
10	0.600	0.041	6.8
15	1.077	0.048	4.4
Slope	0.061		
Correlation coefficient	0.985		

Results and Discussion

To study the linearity, precision, and accuracy of the method, concentrations of clocapramine in both plasma and animal feed were varied from 0 to 15 mg/L for plasma samples, and 0 to 6 g/kg for feed samples. Response ratios were plotted vs clocapramine concentrations. Results of the least squares linear regression analyses (Tables 1 and 2) indicated good linearity (r = 0.985 and 0.986, respectively). Non-zero intercepts are negligible and do not interfere with the detection limits of the method. Typical chromatograms of extracts of plasma and feed spiked with different amounts of clocapramine are shown in Figures 2 and 3, respectively.

Within-day precision of the method was assessed by analyzing plasma and feed samples (n = 15) containing various concentrations of clocapramine. At 5, 10, and 15 mg clocapramine/L plasma, the method yielded coefficients of variation of 3.39, 5.7, and 4.13%, respectively. At animal feed concentrations of 2, 4, and 6 g/kg, the method yielded coefficients of variation (n = 5) of 0.79, 2.16, and 3.7%, respectively (Figure 4). Day-to-day precision was determined by the repeated analysis of the same plasma and animal feed samples on different days. The coefficients of variation were 4.6, 6.8, and 4.4% for plasma samples (n = 15) at 5, 10, and 15 mg/L, respectively, and 0.92, 2.56, and 4.11% for feed samples (n = 5) at 2, 4, and 6 g/kg, respectively.

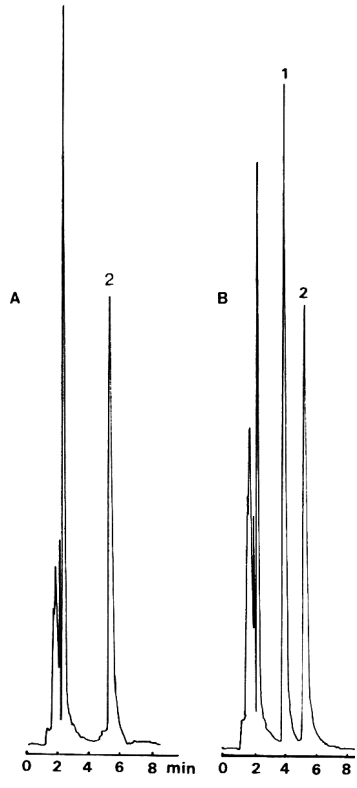


Figure 2. Chromatograms obtained by extracting 1 mL plasma. 1 = Clocapramine, 2 = opipramol. Volume injected 20 μL. A = blank plasma, B = plasma spiked with clocapramine (15 mg/L).

We measured the recovery of clocapramine added to drug-free plasma and to animal feed. The analysis was carried out as described, using free internal standard solution for extraction. Accurately measured aliquots of the final solution were chromatographed and peak areas were determined. Percent recovery was calculated by comparing peak areas of chromatographed extracts with peak areas obtained by direct injection of pure clocapramine. Overall recovery at various concentrations was 90–96%.

Table 2. Linear regression of clocapramine:opipramol response ratio vs clocapramine concentrations (animal feed samples)

Clocapramine concn, g/kg	Peak ht ratio	SD (n = 5)	RSD, %	Area ratio	SD (n = 5)	RSD, %
2	0.410	0.008	2.00	0.314	0.025	0.79
4	0.870	0.009	1.03	0.648	0.014	2.16
6	1.180	0.034	2.90	0.954	0.035	3.70
Slope	0.193			0.159		
Correlation coefficient	0.986			0.991		

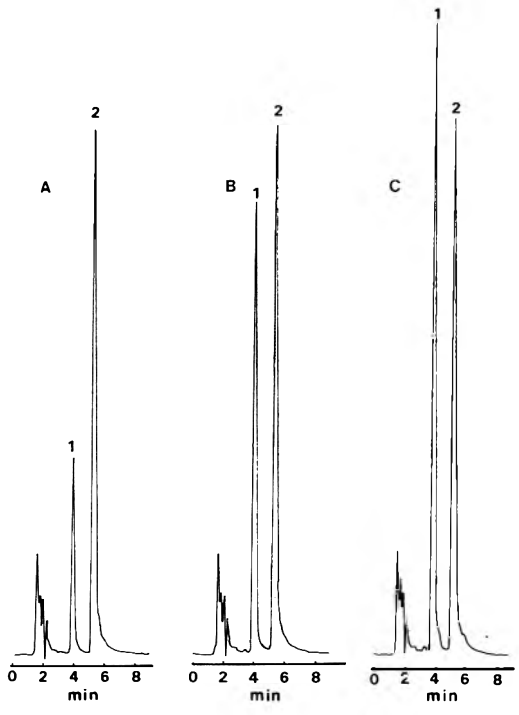


Figure 3. Chromatograms of spiked animal feed standards. A = 0.2%, B = 0.4%, C = 0.6%. 1 = Clocapramine; 2 = opipramol; 2 g feed extracted with 20 mL $CHCl_3$.

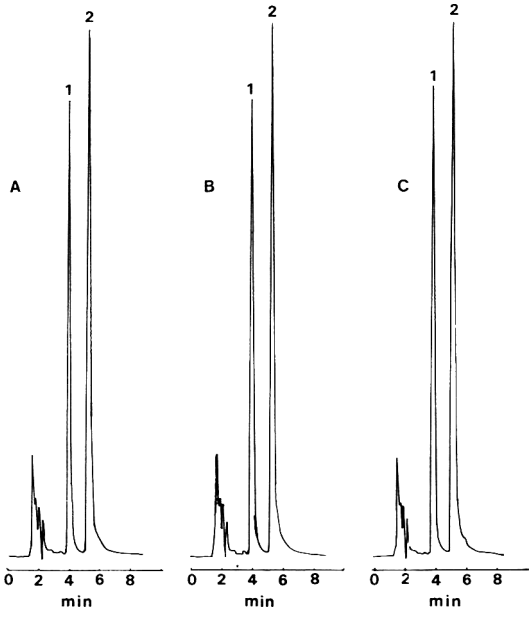


Figure 4. Chromatograms of 3 animal feed extracts (0.2%) showing accuracy of method.

Table 3. Stability of clocapramine added to animal feed [a]

Clocapramine concn, g/kg	Peak ht ratio, clocapramine to opipramol		
	1	2	*t*-test
2	0.412	0.408	0.76
4	0.866	0.858	1.03
6	1.148	1.188	0.20

[a] Samples (n = 5) were analyzed at the beginning of the study (1), and one month later (2). All samples were kept under simulated storage conditions required in toxicological experiments.

This method enabled us to detect as low as 500 ng clocapramine/mL plasma. Detection limits can be lowered by dissolving the extract in 0.1 mL of mobile phase. The detection limit for feed was 30 mg/kg.

No plasma or feed constituents were found with retention times similar to either clocapramine or opipramol, which could cause interference.

The method was used to test the stability of clocapramine in animal feed. Demonstration of stability and homogeneity of the test substance in the dosage form under simulated storage conditions is required before toxicological experiments with animals can be initiated. Results from such studies with clocapramine in feed spiked at 2, 4, and 6 g/kg levels are presented in Table 3. Close agreement at different levels of clocapramine indicates that the mixtures were essentially homogeneous and stable during the test period.

References

(1) Wallace, J. E., & Dahl, E. V. (1967) *J. Forensic Sci.* **12,** 484-496
(2) Moody, J. P., Whyte, S. F., & Naylor, G. Y. (1973) *Clin. Chim. Acta* **43,** 355-359
(3) Faber, P. B., Mulder, C., & Man in't Veld, W. A. (1974) *J. Chromatogr.* **100,** 55-61
(4) Hulshoff, A., & Perrin, J. H. (1976) *J. Chromatogr.* **120,** 65-80
(5) Hucker, H. B., & Stauffer, S. C. (1974) *J. Pharm. Sci.* **63,** 296-297
(6) Wallace, J. E., Hamilton, H. E., Goggin, L. R., & Blum, R. (1975) *Anal. Chem.* **47,** 1516-1519
(7) Gifford, L. A., Tuner, P., & Pare, C. M. B. (1975) *J. Chromatogr.* **105,** 107-113
(8) Belvedere, G., Burti, L., Frigerio, A., & Pantarotto, C. (1975) *J. Chromatogr.* **111,** 313-321
(9) Watson, J. D., & Stewart, M. J. (1975) *J. Chromatogr.* **110,** 389-392
(10) Knox, J. H., & Durand, J. (1975) *J. Chromatogr.* **103,** 311-326

(11) Mellstroem, B., & Eksborg, S. (1976) *J. Chromatogr.* **116,** 475–479
(12) Detaevernier, M. R., Dryon, L., & Massart, D. L. (1976) *J. Chromatogr.* **116,** 475–479
(13) Watson, I. D., & Stewart, M. Y. (1977) *J. Chromatogr.* **132,** 155–159
(14) Van Der Berg, J. H. M., De Ruwe, H. J. J. M., & Deelder, R. S. (1977) *J. Chromatogr.* **138,** 431–436
(15) Watson, J. D., & Stewart, M. J. (1977) *J. Chromatogr.* **134,** 182–186
(16) Brodie, R. R., Chasseaud, L. F., & Hawkins, D. R. (1977) *J. Chromatogr.* **143,** 535–539
(17) Kraak, J. C., & Bijster, P. Y. (1977) *J. Chromatogr.* **143,** 499–512
(18) Mellstroem, B., & Tybring, G. (1977) *J. Chromatogr.* **143,** 597–605
(19) Haefelfinger, P. (1979) *J. Chromatogr. Sci.* **17,** 345–349

Graphite Furnace Atomic Absorption Spectrophotometric Determination of 4-Hydroxy-3-Nitrobenzenearsonic Acid, Other Organic Arsenicals, and Inorganic Arsenic in Finished Animal Feed

GLENN M. GEORGE, LARRY J. FRAHM, and J. PATRICK McDONNELL
Salsbury Laboratories Inc., Pharmaceutical Development and Analysis Department, Charles City, IA 50616

4-Hydroxy-3-nitrobenzenearsonic acid (roxarsone) is administered in animal feed as a growth stimulant over a concentration range of 25–50 ppm. The drug is extracted from 5 g feed with 200 mL aqueous 1.0% ammonium carbonate solution and 5 min of mechanical shaking. Undissolved feed particles are allowed to settle and 1.0 mL aliquot of extract is diluted with 9.0 mL 15% methanol solution. This solution is subjected to sample atomization by a graphite furnace and arsenic detection by atomic absorption spectrophotometry (AAS). Roxarsone recovery from nonmedicated commercial feed fortified at 25 ppm was 103.6% with a relative standard deviation (RSD) of 4.0%. Recovery for 50 ppm fortification was 104.5% (RSD 4.3%). Roxarsone assay results by furnace AAS were compared with results by the current AOAC spectrophotometric method and the AOAC total arsenic method. Results by the 3 methods compare well. The procedure was also used to determine other organic arsenicals and inorganic arsenic in laboratory-fortified feed samples; these recoveries were essentially theoretical.

4-Hydroxy-3-nitrobenzenearsonic acid (roxarsone) stimulates growth and improves feed efficiency in chickens, turkeys, and swine. It also improves pigmentation of chickens and turkeys and aids in controlling swine dysentery when incorporated in finished animal feed. The usual dosage range for chickens and turkeys is 25–50 ppm; for swine the dosage ranges from 25 to 37.5 ppm.

Currently, there are 2 official AOAC methods for determining roxarsone in feeds. One method is a total arsenic determination involving sample digestion by dry ashing, followed by distillation of arsenic as arsine gas into a trapping solution which reacts with the arsenic to produce a colored solution (1–3). Color intensity of the solution is read on a spectrophotometer and the arsenic concentration is determined from a standard curve. Arsenic is then converted by the appropriate factor to roxarsone concentration in the feed.

The second method is a spectrophotometric determination involving the extraction of roxarsone from medicated feed by using dibasic potassium phosphate (4,5). Extract cleanup involves protein precipitation by pH adjustment to 4.5 and centrifugation. The centrifugate is made strongly basic with sodium hydroxide, treated with charcoal, and filtered to remove feed interferences. The resulting clear yellow solution is read in a spectrophotometer at 410 nm and roxarsone concentration is determined from a standard curve.

These methods are accurate and precise, but involve a lengthy sample preparation. In searching for a more rapid assay for roxarsone in finished feed, we investigated the graphite furnace atomic absorption technique. This technique requires very little sample preparation time, with no sample cleanup required before analysis. Sample detection and readout steps require approximately 2 min. Sample introduction into the furnace can be automated. A large daily throughput of samples is possible by using furnace AAS.

METHOD

Principle

Five g finished feed is extracted with aqueous 1% ammonium carbonate. Feed extract is allowed to settle for ca 15 min. A 1 mL aliquot is diluted with 9.0 mL 15% methanol solution and mixed. The feed extract is then analyzed by furnace AAS.

Reagents

(a) *Water.*—Glass-distilled.

(b) *Nitric acid.*—Mallinckrodt, ACS grade.

(c) *Argon.*—Linde purified.

(d) *Nickel nitrate.*—$Ni(NO_3)_2 \cdot 6H_2O$ (Mallinckrodt AR).

(e) *Nickel nitrate solution.*—Approximately 2000 ppm Ni. Dissolve 10.0 g nickel nitrate in water and dilute to 1 L with water.

(f) *Ammonium carbonate.*—Powder, purified (Matheson, Coleman & Bell).

(g) *Methanol.*—Anhydrous, ACS (Matheson, Coleman & Bell).

Received September 9, 1981. Accepted December 4, 1981.

0004-5756/82/6503-0711-09$01.00

(h) *15% Methanol solution.*—Add 150 mL methanol to 1 L volumetric flask, add 5.0 mL HNO_3, dilute to volume with water, and mix.

(i) *Roxarsone standard solution.*—1250 ppm roxarsone (356 ppm As). Accurately weigh 625.0 mg roxarsone reference standard material (Salsbury Laboratories, Inc., Charles City, IA 50616) into 500 mL volumetric flask. Dissolve and dilute to volume with 2% ammonium carbonate.

(j) *12.5 ppm Roxarsone solution.*—3.56 ppm As. Dilute 1 mL roxarsone standard solution to 100 mL with water.

(k) *Control feed extract.*—Using typical nonmedicated poultry or swine ration, prepare extract as described for sample preparation. Test suitability of control feed extract by diluting an aliquot with 15% methanol solution as in sample preparation. Set up AAS system and furnace conditions as described in procedure. It is not necessary to perform the calibration for this test; the absorbance reading is satisfactory. Zero spectrophotometer on 20 μL 15% methanol solution and measure absorbance on 20 μL of this solution. Absorbance <0.030 indicates suitability.

(l) *Working standard.*—Transfer 1.0 mL 12.5 ppm roxarsone solution to 10 mL volumetric flask. Dilute to volume with control feed extract. Transfer 1.0 mL aliquot of this solution to 25 mL Erlenmeyer flask and add 9.0 mL 15% methanol solution. Twenty μL of this working standard is equivalent to 50 ppm roxarsone in the feed sample for weights and volumes given in the procedure.

Apparatus

(a) *Atomic absorption spectrophotometer.*—Perkin-Elmer Model 5000.

(b) *Graphite furnace.*—Perkin-Elmer heated graphite atomizer, Model 400.

(c) *Recorder.*—Houston Instrument, 10 mV full scale.

(d) *Mechanical shaker.*—Burrell, wrist-action.

(e) *Pipets.*—Eppendorf 3130: 10, 20, and 1000 μL.

(f) *Dispensing pipet.*—Lab Industries Repipet, 10 mL capacity set to deliver 9.0 mL 15% methanol solution.

Preparation of Sample

Grind sample in a high-speed blender to pass U.S. No. 20 sieve (ca 3 min) and mix thoroughly. Weigh 5.0 g ground sample into 250 mL volumetric flask or 300 mL Erlenmeyer flask. Add 2.0 g ammonium carbonate powder to flask. Add 200.0 mL water to flask, place on mechanical shaker, and shake vigorously 5 min at room temperature. Remove flask from shaker and let feed particles settle ca 15 min.

Transfer 1.0 mL aliquot to 25 mL Erlenmeyer flask and add 9.0 mL 15% methanol solution. Repeat this step on reagent blank and on standard fortified control feed extract equivalent to 50 ppm roxarsone in feed. Samples and standard are now ready for furnace AAS analysis. If additional sample dilutions are required to bring roxarsone concentrations of feed into concentration range of standard, dilutions must be made with 1.0% ammonium carbonate. Do not use 15% methanol solution to make additional dilutions. If an additional sample dilution is made, multiply results by appropriate dilution factor.

AAS Conditions

Set up graphite furnace and spectrophotometer according to following conditions and allow 30 min warmup time. Operating conditions: program, 1 (No. 1 on magnetic card also); lamp, arsenic EDL operated at 8 watts, properly aligned; lamp position, 1; lamp current, 0 ma; wavelength, 193.7 nm; slit, 0.7 nm bandpass, low position; readtime, 2 s; mode, AA-BG; readout, concentration; signal, peak height on instrument display and absorbance on recorder; standard 1, S1 = 50.0 ppm (μg/g) roxarsone (use 3 digits), S2 and S3 not used.

Furnace Conditions

Install furnace assembly in AAS system and align as in manufacturer's instructions. Operating conditions: furnace tube, pyrolytically coated tube; water flow, 1–2 L/min to cool furnace (on humid days, preheating water may be required to prevent condensation); argon pressure, 35 psi; ON/OFF switch, ON; gas control, ON.

Step 1 (drying): temperature, 100°C; ramp time, 10 s; hold time, 50 s.

Step 2 (charring): temperature, 1000°C; ramp time, 10 s; hold time, 30 s.

Step 3 (atomizing): temperature, 2300°C; ramp time, 0 s; hold time, 5 s; recorder, ON; read, ON; stop flow, ON.

Step 4 (burnout): temperature, 2400°C; ramp time, 0 s; hold time, 5 s; recorder, OFF; read, OFF; flow, 300 mL/min (stop flow OFF).

Matrix Modification

Each time a blank, sample, or standard is injected into furnace tube, first inject 10 μL 2000 ppm nickel solution.

Calibration

Zero instrument on reagent blank (15% methanol diluting solution).

Inject 20 μL 50.0 ppm roxarsone-fortified control feed standard (working standard) into furnace tube. Run furnace through its cycle and note reading on instrument display. Repeat standard injection until stable readings are obtained (2 or 3 injections). Now press switch S1 to establish existing absorbance readings on the display as equivalent to 50.0 ppm roxarsone in medicated feed.

Once satisfactory results are observed, instrument is ready for sample analysis. Inject 20 μL sample solution into furnace tube, run furnace through its cycle, and record reading from instrument display in ppm roxarsone.

Calibration of instrument should be checked periodically to monitor changes in calibration that may occur as assay progresses.

Calculations

ppm Roxarsone = instrument reading (ppm roxarsone) × DF where DF is appropriate dilution factor if additional dilution was used in preparation of sample.

Results and Discussion

Use of the graphite furnace for sample atomization allows detection of very low levels of arsenic. The lower limit of detection for arsenic by furnace AAS is approximately 0.0002 mg/L, using an electrodeless discharge lamp and background correction (6).

This low level detection permits analysis of feeds for organic arsenical (roxarsone) medication with little sample preparation. This was not possible by flame AAS because the feed sample extract contains less arsenic than the minimum detection levels. With a minimum amount of sample preparation required and an approximately 2 min cycle with furnace AAS, the sample throughput per day can be rather large (over 100 samples).

The AAS and furnace conditions were studied first and a general set of conditions was developed and monitored to ensure optimum conditions as the method evolved.

Background correction is necessary for low level arsenic analysis, especially when using the graphite furnace, because of the low UV wavelength (193.7 nm) used. Background can cause high positive values for the reagent blank and control feed extracts, which result in an increase in the detection limits. Background correction improves the accuracy and precision especially with samples such as feed extracts which have not had the benefit of cleanup.

Sample drying in the furnace tube is a very critical step; the sample must dry without bubbling or spattering for accurate and precise results. By monitoring the signal generated on the recorder during the drying cycle, we observed that the drying step required approximately 50 s to dry the sample completely for a brand new furnace tube. After about 100 firings, the drying time could be reduced to approximately 40 s.

The charring or ashing step was studied from 500 to 1500°C by running the furnace through its complete cycle with sample injections. Acceptable results (low blank feed values) were observed for charring temperatures from 600 to 1100°C. The 1000°C temperature was chosen because the precision of assay results was best at that temperature. Temperatures above 1200°C showed arsenic losses due to premature volatilization.

The atomization step was studied from 2000 to 2700°C. The lowest atomization temperature that can be used successfully is 2200°C. Temperatures up to 2700°C may be used; however, this is not necessary if complete atomization can be accomplished at the lower temperature. Higher atomization temperatures shorten the useful life of the furnace tubes. At the 2300°C temperature selected, a tube is normally good for more than 200 firings.

A burnout step was instituted to make sure that memory would not become a problem and also to minimize carbon buildup within the tube (7). Burnout is carried out with full flow of purge gas, and at a temperature about 100°C higher than for atomization. Here again, higher temperatures shorten tube life.

Using the conditions described for roxarsone analysis, response for the 50 ppm standard is about 0.300 absorbance unit. The furnace tube will maintain this response for many firings; however, response will decrease slightly as the tube is used. The furnace tube should be replaced when erosion around the outside of the tube entrance becomes extensive.

The present AOAC (4, 5) extraction procedure for roxarsone specifies 2% dibasic potassium phosphate. Furnace AAS response for arsenic in this matrix is very poor, only about 25% of the expected response. It appears that the phosphate interferes with the action of the matrix modifier (nickel) and the arsenic is prematurely volatilized in the furnace. Aqueous phosphate extraction was abandoned, and several other basic

systems, sodium hydroxide, potassium hydroxide, ammonium hydroxide, and ammonium carbonate, and one organic base (diethylamine) were investigated.

Sodium and potassium hydroxide extractions, investigated at the 0.1% level, produced variability of results and some unexplained high results. Diethylamine extraction was only about 10% complete. Other organic solvents have been investigated earlier and were ineffective for roxarsone extraction from feed.

Extraction of 5 g of feed with 200 mL 1% ammonium carbonate produced acceptable results for roxarsone-medicated feeds, with no further sample treatment other than sample dilution. Extraction with ammonium carbonate at concentrations from 0.5 to 2.5% was investigated. At the 2.5% level, a general decrease in response was observed; otherwise results did not appear to be affected, provided the standard and samples were maintained in the same matrix. The 1% level was chosen as optimum.

With the extracting solvent chosen, a linearity of response study was undertaken to establish the linear working range for roxarsone in medicated feeds in the ammonium carbonate matrix. Purified reference standard roxarsone was used as the arsenic source. The concentrations studied ranged from 0 to 100 ppm roxarsone equivalent in 5 g of feed per 200 mL ammonium carbonate extractant. Response was 0.004A for 0 ppm, 0.153A for 25 ppm, 0.328A for 50 ppm, 0.536A for 75 ppm, and 0.625A for 100 ppm. Response is linear up to 75 ppm. At 100 ppm, the response is diminished slightly; however, results are still satisfactory. Above 100 ppm, an additional dilution with 1% ammonium carbonate is necessary. The appropriate dilution factor (DF) must be used in calculating results.

Because the sample matrix is a direct feed extract with no sample cleanup, it contains a considerable amount of co-extracted extraneous material. Furnace conditions had to be optimized for the feed extract containing roxarsone, not just for a roxarsone standard solution. Occasionally during the drying step, the feed extract emitted a frying sound when the tube reached 100°C and this continued until the water was completely removed. When this happened, roxarsone results for medicated feeds were always high compared with the external standard prepared with 1% ammonium carbonate. Adding methanol to the diluting solution reduced this problem.

Matrix modification is required for arsenic analysis to stabilize the arsenic and make it less volatile, allowing charring at temperatures up to 1200°C with no loss of arsenic. If no matrix modifier is used, arsenic will start to volatilize at 300–400°C. Charring is especially important in feed analysis because it destroys organic material that could interfere in the atomizing step. Feed extracts with no sample cleanup have a matrix high in organic material. Several metals could be used as matrix modifiers; however, nickel is highly recommended and is widely used (8–10). It has been established (G. M. George et al., Salsbury Laboratories, Charles City, IA (1980), unpublished data) that the nickel nitrate solution should be added separately to the furnace tube and not mixed into the sample. To minimize confusion, one should develop the habit of adding the nickel to the furnace tube first and then adding the sample. However, order of addition of nickel and sample is not important.

The 20 μL sample injection volume appears to be optimum because this volume provides adequate sample to produce the desired response. Injection volumes of 25 and 50 μL provided more response, but response difference between a 25 ppm and 50 ppm roxarsone feed decreased, indicating a change in linearity.

Under the analytical conditions of this method, a very small amount of arsenic is being detected in the final sample solution. Sample size and extraction volumes specified appear to be optimum. If a larger feed sample is extracted or a larger aliquot is diluted before injection, the higher arsenic concentration results in a nonlinear response.

The stop flow purge gas mode used during the atomization step produced higher instrument readings and also provided more reproducible readings from repeated injections of the same sample.

A study was performed to determine if the arsenic source (organic or inorganic) had any effect on results. Feeds and standards at the 50 ppm roxarsone equivalent level were prepared using roxarsone and arsenic trioxide as arsenic sources and were assayed by the described procedure using roxarsone as the reference standard arsenic source. Theoretical recovery was observed for the arsenic trioxide-fortified samples. Furnace AAS response is equivalent for different chemical forms of arsenic. Reference standard roxarsone was chosen as the standard arsenic source for analysis of roxarsone-medicated feeds.

This procedure uses a roxarsone-fortified control feed extract as the working standard for calculation of the drug concentration in medi-

cated feed samples. For AAS determinations, Slavin (11) and Welz (12) recommend matching the standard matrix to the sample matrix content when there is >0.1% dissolved solids in the sample. We did this by fortifying a control feed extract with the proper concentration of roxarsone. This technique helps to maintain accuracy and precision of results. This match is an attempt to make the physical and chemical properties of the 2 solutions similar (13, 14).

The control feed used to prepare the reference standard must be shown to be acceptable for this purpose. The absorption value observed for control feed carried through the procedure should not be more than 0.030 absorbance unit above the reagent blank to be acceptable for use in reference standard preparation.

With the operating conditions established, a recovery and reproducibility study was performed on a control commercial poultry mash feed formulation (Supersweet) fortified in the laboratory at 25 and 50 ppm roxarsone. At the 25 ppm fortification level, roxarsone recoveries ranged from 24.1 to 27.6 ppm (21 injections) with a mean of 25.9 ppm. The standard deviation (SD) was 1.03 and the coefficient of variation (RSD) was 4.0%. Mean percent recovery was 103.6. Results at the 50 ppm level were: recovery range, 49.3–56.4 ppm (28 injections); mean, 52.3 ppm; SD, 2.2; RSD, 4.3%; mean percent recovery, 104.5. These values contain no correction for blank (control feed) values, nor do any recovery values discussed in this paper. Satisfactory recovery and precision were observed for laboratory-medicated feed.

Several nonmedicated control feed samples received from customers in various regions of the United States were analyzed by furnace AAS and by the AOAC total arsenic assay (1). Results were comparable. The furnace AAS procedure showed a blank calculated as roxarsone of 1.8 ppm (0.51 ppm total arsenic) and the AOAC total arsenic method showed a blank calculated as roxarsone of 1.4 ppm (0.40 ppm total arsenic) (Table 1).

To study roxarsone recovery in different feed formulations by furnace AAS, the nonmedicated customer control feeds assayed earlier to establish background were fortified in the laboratory with 25 and 50 ppm roxarsone and subjected to analysis. Results (Table 2) show a mean recovery for the 25 ppm level of 103.1% and for the 50 ppm level of 104.8%. Feed No. S-141 showed a control value of 9.3 ppm roxarsone and recovery at the 50 ppm fortification level reflects the high blank. This feed was added to the series to show that results by this method will reflect a high blank value.

Table 1. Determination of roxarsone (ppm) in nonmedicated control feed by furnace AAS and AOAC total arsenic method

Sample No.	Furnace AAS	Total As method[a]
Control 1	1.7	3.8
2	2.5	—
3	3.6	—
4	2.1	—
1494	2.5	1.3
1495	2.7	0.0
1496	1.3	0.0
1497	3.5	0.6
1498	0.5	0.9
1499	0.4	1.0
1500	0.8	1.1
1673	0.9	2.6
1674	1.9	1.0
1675	2.9	2.0
1788R	0.4	0.0
1789R	0.4	2.0
S145	2.3	3.7
Mean	1.8	1.4
SD	1.09	1.25

[a] AOAC method **42.005–42.010** (ref. 1).

A random selection of customer roxarsone-medicated feeds were analyzed by furnace AAS, the AOAC spectrophotometric method (5), and the AOAC total arsenic method (1). Results by the 3 methods (Table 3) compare well. Analysis by the Duncan Multiple Range Test showed no significant difference between the furnace AAS and either of the AOAC methods at the 95% confidence level. The feeds used in this study were customer feeds sent to the feed laboratory

Table 2. Recovery of roxarsone (ppm) from fortified customer control feeds by furnace AAS

Sample No.	Nonmedicated	25 ppm fortification	50 ppm fortification
1494	2.5	24.2	53.8
1495	2.7	26.9	50.4
1497	3.5	25.9	51.5
1498	0.5	25.4	52.7
1499	0.4	23.9	53.3
1500	0.8	25.0	51.8
1673	0.9	28.0	51.4
1674	1.9	25.6	53.3
1675	2.9	26.9	52.5
1788R	0.4	25.4	49.4
1789R	0.4	25.4	48.8
S141[a]	9.3	—	59.6
S145	2.3	26.8	52.3
Mean	2.2	25.8	52.4
Rec., %		103.1	104.8

[a] Sample No. S141 was not a control feed; some roxarsone was present.

Table 3. Comparison of roxarsone determination (ppm) in customer roxarsone-medicated feeds by 3 assay methods

Sample No.	Furnace AAS	Colorim.[a]	Total As method[b]
		Low Levels	
1451	18.1	17.0	17.1
1457	8.8	12.0	8.7
1459	9.0	12.0	8.3
1460	15.0	15.0	13.0
1578	22.5	17.5	20.5
1588	17.6	16.0	14.4
1589	18.2	16.5	18.2
1623	15.8	18.0	14.7
1670	12.3	15.5	12.3
1676	11.9	13.5	11.9
1679	14.4	14.5	13.3
1682	17.9	16.5	16.1
		High Levels	
S91	26.3	25.0	25.2
S92	54.2	50.2	46.3
S133	52.2	51.0	48.6
S140	49.0	49.0	44.6
S146	51.6	49.0	48.6
S155	40.0	40.0	36.0
S165	42.0	42.0	43.3
S167	46.0	41.0	43.2
S202	40.5	37.0	33.1
S205	29.3	31.0	31.4
S212	29.3	29.0	33.0
S224	21.2	23.0	22.7
S225	53.8	53.0	54.1
S226	40.8	43.0	—
S165	38.6	45.0	—
S230	19.6	19.0	—
S168	49.7	45.0	—
S227	46.5	45.0	—
S229	54.7	47.0	—

[a] AOAC method **42.153–42.160** (ref. 5).
[b] AOAC method **42.005–42.010** (ref. 1).

for assay; the true amount of roxarsone in these samples is not known; hence, recovery cannot be calculated.

Because the standard curve indicates linear response up to approximately 100 ppm roxarsone in feed, a group of medicated customer feeds that had been previously assayed by furnace AAS were fortified with an additional 25 and 50 ppm roxarsone and analyzed again by furnace AAS. This fortification raises the roxarsone concentration above the normal use level and also provides the analytical method with the added burden for recovery of roxarsone at elevated levels. Mean recoveries (Table 4) were 99.9 and 102.7% at the 25 and 50 ppm fortification levels, respectively.

Furnace AAS results were compared with results by AOAC methods on customer premix samples in the concentration range 0.10–1.50% roxarsone (exact concentrations unknown). Results from the 3 methods (Table 5) are comparable for the premix concentrations assayed. Assay by furnace AAS at premix levels requires an adjustment in sample size and dilutions. In the range 0.1–2.0%, a 0.5 g sample is extracted with 200 mL 1% ammonium carbonate. This weight change is equivalent to a dilution factor (DF) of 10. Additional dilutions of $1/4$ to $1/40$ with 1% ammonium carbonate are necessary in the 0.1–2.0% range. For the final sample preparation, 9.0 mL 15% methanol solution is added to 1 mL of the dilution. Results by the 3 assay procedures were analyzed by the Duncan Multiple Range Test. Comparison of the furnace AAS method with the AOAC spectrophotometric

Table 4. Recovery of roxarsone from fortified customer roxarsone-medicated feeds by furnace AAS

		Furnace AAS[b]				
			25 ppm added		50 ppm added	
Sample No.	Colorim.[a] ppm	Initial, ppm	Total found, ppm	Rec., %	Total found, ppm	Rec., %
1591	27.0	31.0	53.0	88.0	85.6	109.2
1611	24.0	25.5	51.4	103.6	75.1	99.2
1636	25.0	25.8	48.8	92.0	72.5	93.4
1638	26.0	26.8	53.6	107.2	76.7	99.8
S146	49.0	47.4	76.7	117.2	100.2	105.6
S155	40.0	39.9	64.6	98.8	92.6	105.4
S165	42.0	47.2	75.4	112.8	99.9	105.4
S168	45.0	48.0	72.5	98.0	99.9	103.8
S202	37.0	40.1	67.1	108.0	94.5	108.8
S226	43.0	44.4	68.7	97.2	96.9	105.0
S227	45.0	48.6	70.7	88.4	100.0	102.8
S229	47.0	55.4	77.4	88.0	102.2	93.6
Mean rec., %				99.9		102.7

[a] AOAC method **42.153–42.160** (ref. 5).
[b] Furnace AAS results are averages of duplicate analyses.

Table 5. Comparison of results of determination of roxarsone (%) in premixes by 2 AOAC methods

Sample No.	Furnace AAS	Colorim.[a]	Total As method[b]
1592	1.120	1.160	1.340
1600	0.135	0.110	0.102
1601	0.185	0.173	0.151
1603	0.176	0.140	0.161
1604	0.162	0.129	0.118
1607	1.420	1.470	1.550

[a] AOAC method **42.153–42.160** (ref. 5).
[b] AOAC method **42.005–42.010** (ref. 1).

method or the AOAC total arsenic method showed no significant difference between methods at the 95% confidence level.

No attempt has been made to study method performance in mash vs pelleted feeds. More than 50% of the feeds received in our Feed Analysis Laboratory are pelleted, and the procedure has been used to analyze them. Results do not indicate any bias in the method. Comparison of results by furnace AAS with 2 proven methods (1, 5) indicates satisfactory performance for the ammonium carbonate extraction in both mash and pelleted feeds.

Other means of reducing matrix interferences were also investigated to determine if accuracy and precision could be improved.

The L'Vov platform (15–18) was evaluated. Using the L'Vov platform in the furnace tube required longer ramping as well as longer hold times at higher temperatures to satisfactorily complete the drying and charring cycles. For the drying cycle, a 220°C temperature with a 30 s ramp time and 40 s hold time was required to successfully complete the sample drying. For charring, a 1200°C temperature was required to do a complete sample char with no change in the ramp or hold times.

Performance of the L'Vov platform was studied by repeated analyses of a poultry control mash-type feed laboratory-fortified at 25 and 50 ppm roxarsone. At the 25 ppm level, roxarsone recoveries ranged from 24.3 to 27.4 ppm (20 injections), with a mean of 25.8 ppm. The standard deviation (SD) was 0.81 and the relative standard deviation (RSD) was 3.1%. Mean percent recovery was 103.2. Results at the 50 ppm level were: recovery range, 49.3–54.0 ppm (20 injections); mean, 50.7 ppm; SD, 1.23; RSD 2.4%, mean percent recovery, 101.4. The L'Vov platform improved the accuracy and precision slightly when compared with results omitting its use, however, improvement was minimal and, therefore, the L'Vov platform is not recommended for use in routine analyses.

The use of oxygen as a charring aid was studied by introducing oxygen into the pyrolytically coated furnace tube after the initial sample-drying step (7, 19, 20). This was done at 600°C to prevent rapid deterioration of the furnace tube. The tube was then flushed with argon before proceeding to the 1000°C charring step. No changes were made for the atomization and burnout steps.

The method was evaluated under these conditions on 25 and 50 ppm laboratory-fortified control mash-type feed. At the 25 ppm level, roxarsone recoveries ranged from 24.9 to 28.8 ppm (10 injections) with a mean of 26.1 ppm (SD, 0.82, RSD, 3.2%). Mean percent recovery was 104.4%. Results at the 50 ppm level were: recovery range, 48.0–56.2 ppm (12 injections); mean, 51.5 ppm; SD, 2.20; RSD, 4.3%; mean percent recovery, 103.0%. These results are very similar to results obtained without the use of oxygen in the char step (mean percent recoveries 103.6 and 104.5% at the 25 and 50 ppm levels, respectively). The addition of oxygen to the char step had no apparent effect on the analyte signal except a slight loss in sensitivity was observed (13). Because this modification showed no improvement in precision and accuracy, use of oxygen is not recommended.

The addition of organic acid matrix modifiers (21, 22) to the feed sample matrix was investigated as another means of improving assay results. Two organic acid matrix modifiers, ascorbic acid (21) and citric acid (22) were added to the feed extract. Their use caused the arsenic AAS readings to become erratic. We did not investigate this concept further and do not recommend using organic acids as matrix modifiers for roxarsone determinations in mixed feeds.

With the assay conditions established for graphite furnace AAS, a limited amount of data was generated for furnace analysis of feeds fortified with other arsenic-containing compounds. Five separate portions of a nonmedicated commercial poultry mash starter ration were fortified in the laboratory to contain 100 ppm arsanilic acid, 187.5 ppm nitarsone, or 250 ppm carbarsone, or 10 ppm each for arsenic from arsenic trioxide and sodium arsenate. Each feed sample was extracted as described under sample preparation. The prepared samples were then subjected to graphite furnace analysis using conditions described for the regular pyrolytically coated tube and conditions for use of the L'Vov

Table 6. Organic and inorganic arsenic determinations in fortified feed samples by furnace AAS with pyrolytically coated tube

	Arsanilic acid, 100 ppm		Nitarsone, 187.5 ppm		Carbarsone, 250 ppm		Arsenic trioxide, 10 ppm		Sodium arsenate, 10 ppm	
Assay No.	Dil	Found, ppm	Dil	Found, ppm	Dil	Found, ppm	Dil	Found, ppm	Dil	Found, ppm
1	2	92.3	2.5	186.5	5	257.8	0	9.8	0	9.8
2	2	92.5	2.5	183.3	5	251.6	0	10.6	0	10.0
3	2	97.3	2.5	184.4	5	246.2	0	9.4	0	10.1
4	2	92.8	2.5	176.9	5	225.6	0	9.2	0	9.4
Mean		93.7		182.8		245.3		9.7		9.8
Rec., %		93.7		97.5		98.1		97.0		98.0

platform. Because the 3 organic arsenicals analyzed contain more arsenic than does roxarsone at the recommended use levels in feed, a further sample dilution was necessary to put the arsenic concentration in the linear response range for the instrument. The sample dilutions were made with 1% ammonium carbonate before the final dilution with 15% methanol solution. Results (Table 6) show that recovery of the arsenic added to these feed samples ranged from 93.7 to 98.1%.

Analytical results by furnace AAS using the L'Vov platform for feed samples fortified with organic and inorganic arsenic are shown in Table 7. Recovery of added arsenic ranged from 98.7 to 103%.

The standard used for the above samples was the 50 ppm roxarsone-fortified control feed extract standard as described under sample preparation. For determining these other arsenic-containing compounds, the instrument was programmed to read ppm arsenic. Therefore, the concentration readout was programmed to read 14.24 ppm arsenic (50 ppm roxarsone × 28.48% arsenic = 14.24 ppm arsenic). The arsenic concentration observed for each of the fortified samples was then converted by the appropriate factor to the concentration of each organic arsenical assayed (arsanilic acid = 2.90, nitarsone = 3.30 and carbarsone = 3.47).

The recovery study on the above organic arsenicals and inorganic arsenic was very limited. However, the recovery observed for the different forms of arsenic in the fortified feed was near theoretical. This furnace AAS assay is based on a total arsenic response and is nonspecific as to the form of arsenic present. The assay uses an extraction procedure for removing arsenic or arsenicals from feed. Results show that the 1% ammonium carbonate is adequate for extracting arsenic from feed for each of the different forms of arsenic studied. However, conditions of feed extraction and instrumental parameters have specifically been optimized for the determination of roxarsone in finished animal feeds. While results for the other arsenicals and the inorganic arsenic were satisfactory, conditions have not been optimized for arsenical compounds other than roxarsone.

Experimentation on optimizing detection conditions has shown that the assay procedure is rugged. A rather wide range of furnace parameters will produce satisfactory results. Use of L'Vov platform did produce a slight im-

Table 7. Organic and inorganic arsenic determinations in fortified feed samples by furnace AAS using the L'Vov platform

	Arsanilic acid, 100 ppm		Nitarsone, 187.5 ppm		Carbarsone, 250 ppm		Arsenic trioxide, 10 ppm		Sodium arsenate, 10 ppm	
Assay No.	Dil	Found, ppm	Dil	Found, ppm	Dil	Found, ppm	Dil	Found, ppm	Dil	Found, ppm
1	0	95.5	2.5	184.4	5	260.3	0	9.6	0	9.6
2	0	95.1	2.5	188.3	5	252.9	0	10.7	0	10.9
3	2	101.9	2.5	188.9	5	258.5	0	10.5	0	10.6
4	2	102.5	2.5	181.7	5	255.9	0	9.9	0	10.0
Mean		98.7		185.8		256.9		10.2		10.3
Rec., %		98.7		99.1		102.8		102.0		103.0

provement in precision of assay results, but higher temperatures were required for drying and charring of sample. The L'Vov platform is not recommended for routine use. The introduction of oxygen into the char step to burn off interferences did not improve results nor did the quality of data decrease appreciably. The addition of organic acids to the sample matrix did not improve results. The method of sample preparation and the instrumental parameters used for roxarsone also produced satisfactory results for the analysis of arsanilic acid, nitarsone, carbarsone, arsenic trioxide, and sodium arsenate in laboratory-fortified feed samples.

It should be understood that the instrumental parameters described are optimum for the Perkin-Elmer equipment used. An analyst using other equipment may find it necessary to optimize these instrumental parameters for that particular equipment.

In conclusion, a procedure for the determination of roxarsone in finished animal feeds has been developed and evaluated using graphite furnace sample atomization and AAS detection. Method performance was satisfactory for a wide concentration range (0–100 ppm) in finished feeds and also at premix concentrations with adjustments made for sample size and dilution. The method is accurate (104% recovery) and precise (4.1% RSD) at the 25 and 50 ppm roxarsone medication levels. The procedure is also capable of successfully determining other organic arsenicals used as feed additives, as well as inorganic arsenic.

The overall accuracy for inorganic arsenic in a feed matrix is 100.1% recovery with an overall precision (RSD) of 5.19%.

The standard deviation for arsenic (28.48% As in roxarsone) in a nonmedicated feed matrix calculated from the control feed data (Table 1) is 0.31 ppm. A commonly accepted calculation of lower limit of detection (lld) is 2 standard deviations of background noise above zero (23). The lld for this method is estimated to be 0.62 ppm As which is slightly lower than the 0.71 ppm lld for arsenic by the AOAC total arsenic method.

References

(1) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **42.005–42.010**
(2) Morrison, J. L. (1961) *J. Assoc. Off. Agric. Chem.* **44,** 740–741
(3) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **25.012–25.013**
(4) George, G. M., & Morrison, J. L. (1971) *J. Assoc. Off. Anal. Chem.* **54,** 80–85
(5) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, sec. **42.153–42.160**
(6) Beaty, R. E. (1978) *Concepts, Instrumentation and Techniques in Atomic Absorption Spectrophotometry,* Perkin-Elmer Corp., Norwalk, CT
(7) Delves, H. T., & Woodward, J. (1981) *At. Spectrosc.* **2,** 68–70
(8) Ediger, R. D. (1975) *At. Absorpt. Newsl.* **14,** 5
(9) Stein, V. B., Canelli, E. & Richard, A. J. (1980) *At. Spectrosc.* **1,** 133–137
(10) Srydlowski, F. J. (1977) *At. Absorpt. Newsl.* **16,** 60–63
(11) Slavin, W. (1968) *Atomic Absorption Spectroscopy,* Vol. 25, Interscience Publishers, New York, NY, pp. 64, 72
(12) Welz, B. (1976) *Atomic Absorption Spectroscopy,* Aulf-Weinheim, New York, NY, p. 100
(13) Hobert, J., & Candelaver, R. M. (1980) *At. Spectrosc.* **1,** 90–93
(14) Thompson, D. D., & Allen, R. J. (1981) *At. Spectrosc.* **2,** 53–58
(15) Slavin, W., & Manning, D. C. (1979) *Anal. Chem.* **51,** 261–265
(16) Gregorire, W. M., & Chakrabarte, C. L. (1977) *Anal. Chem.* **49,** 2018–2028
(17) Hinderberger, E. J., Kaiser, M. L., & Koirtyahann, S. R. (1981) *At. Spectrosc.* **2,** 1–7
(18) Fernandez, F. J., Beaty, M. M., & Barnett, W. B. (1981) *At. Spectrosc.* **2,** 16–21
(19) Beaty, M., Barnell, W., & Grobenski, Z. (1980) *At. Spectrosc.* **1,** 72–77
(20) Beaty, R. D., & Cooksey, M. M. (1978) *At. Absorpt. Newsl.* **17,** 53–58
(21) Gueriremort, R. (1980) *Anal. Chem.* **52,** 1574–1578
(22) Hydes, D. J. (1980) *Anal. Chem.* **52,** 959–963
(23) Kahn, H. L. (1968) *Adv. Chem. Ser. No. 73,* American Chemical Society, Washington, DC, pp. 191–192

FOOD ADDITIVES

Gas-Liquid Chromatographic-Thermal Energy Analyzer Determination of *N*-Nitrosodimethylamine in Beer: Collaborative Study

NRISINHA P. SEN, STEPHEN SEAMAN, and MIKELIS BICKIS
Health and Welfare Canada, Food Directorate, Health Protection Branch, Ottawa, Ontario, Canada K1A 0L2

Collaborators: M. Castegnaro; G. Ellen; T. Fazio; W. Fiddler; D. H. Fine; W. C. Herwig; P. Issenberg; A. B. Lacey; R. Massey; T. O'Brien; R. Scanlan; G. Thomas; J. Thomsen

The GLC/TEA method for *N*-nitrosodimethylamine (NDMA) in beer was studied collaboratively by 13 laboratories from 7 countries. Collaborators were asked to analyze a total of 10 randomly labeled samples of beer consisting of the following duplicates: a naturally contaminated commercial beer; a beer extremely low (ca 0.1 ppb) in NDMA; and the low NDMA beer spiked with 0.5, 1.9, and 5.0 ppb NDMA. The pooled repeatability and reproducibility coefficients of variation (CV) for all samples were 17% and 27%, respectively. However, when data from 2 laboratories (outliers) were omitted, the corresponding CV values improved considerably (11% and 15%, respectively). Variance analysis showed the presence of a significant laboratory-sample interaction when all data were used for analysis, but this interaction disappeared when data from the 2 outlying laboratories were excluded. The pooled percent recovery of the overall method (omitting outliers) was 101.4 ± 3.5. All the laboratories detected NDMA in the low NDMA beer. The method was adopted official first action.

Traces of *N*-nitrosodimethylamine (NDMA), a potent carcinogen, have been reported to occur in certain types of alcoholic beverages, especially beer and ale (1). Studies in Germany (2) and other countries (1) have shown that malt is the source of the contamination. As a result of improved malt-drying techniques introduced during the past 2 years, levels of NDMA in both malt and beer have decreased significantly (1). Current average levels of NDMA in various beers and ale in Canada (3) and the United States (4) have been reported to be 0.4 ppb and 0.5–1 ppb, respectively.

At the 1980 annual meeting of the AOAC, the Associate Referee reported (5) a sensitive and accurate method for determination of NDMA in beer and ale as low as 0.1 ppb. This paper describes the results of the collaborative study of that method.

Received September 1, 1981. Accepted December 15, 1981.

This report of the Associate Referee, N. P. Sen, 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 C and was adopted by the Association. See the report of the committee, March 1982 issue, for detailed recommendations.

N-Nitrosodimethylamine (NDMA) in Beer
Gas-Liquid Chromatographic Method
Official First Action
Method II

10.C10 *Principle*

Sample is treated with sulfamic acid and HCl, and *N*-nitrosodipropylamine (NDPA) is added as internal std. Soln is made alkaline, and NDMA is sepd by distn and detd by GLC with thermal energy analyzer (TEA) detector.

Caution: *N*-Nitrosamines are potent carcinogens; take adequate precaution to avoid exposure. Carry out all steps, wherever possible, in well ventilated fume hood and wear protective gloves while handling nitrosamine stds Use mech. pipetting aids for measuring all solns. Use sep. pipetting device for measuring stds and mark it appropriately; do not use it for pipetting other reagents. Because these compds are highly photolabile, all work should be carried out under subdued light. Destroy all nitrosamine stds by boiling with HCl, KI, and sulfamic acid before disposal.

10.C11 *Reagents*

(a) *Dichloromethane.*—Distd in glass. Test each bottle before use: Conc. 200 mL to 1 mL as described under *Concentration* and then analyze 10

0004-5756/82/6503-0720-10$01.00

μL aliquot by GLC-TEA. Test must show absence of NDMA.

(b) *Sodium sulfate.*—Anhyd. (granular). Test each bottle as follows: Dissolve 40 g in 50 mL H_2O, add 2 mL 10N KOH, ext with two 50 mL portions of CH_2Cl_2, dry ext over anhyd. Na_2SO_4 as described later, conc. to 1 mL, and analyze 10 μL aliquot by GLC-TEA. Ext must be free of NDMA.

(c) *Distilled or deionized water.*—Test as follows: Take 50 mL H_2O, add 2 mL 10N KOH, ext with two 50 mL portions of CH_2Cl_2, and test for NDMA contamination as under (b). If test under (b) is neg. (which includes 50 mL H_2O), there is no need to test H_2O sep.

(d) *Boiling aids.*—Boileezers® (Fisher Scientific Co., Catalog No. B-365), or equiv.

(e) *Sulfamic acid.*—10% in H_2O; store at 4°.

(f) *KOH.*—10N and 3N; store in polyethylene bottles.

(g) *NDMA.*—(*1*) *Stock soln, 10 mg/mL.*—Accurately weigh (±0.0001 g) ca 100 mg in 10 mL vol. flask (with polyethylene stopper), dil. to mark with CH_2Cl_2, and mix well. Store at −20°, and warm to room temp. in the dark before use. Prep. fresh stock soln once a year. (*2*) *Dilute solns.*—By serial dilns (using ≥1 mL pipets) (*1*, above), prep. the following NDMA solns in CH_2Cl_2: 500, 200, 100, 40, 20, 10, and 5 ng/mL. Store at −20° and warm to room temp. in dark before use. Prep. fresh dil. stds once a month.

(h) *N-Nitrosodi-n-propylamine (NDPA) stds.*—As described above, weigh and prep. soln contg 250 ng NDPA/mL anhyd. EtOH. Note: Dild stds available from com. firms are acceptable. Always use appropriate solvs (CH_2Cl_2 for NDMA and anhyd. EtOH for NDPA) for dilns.

10.C12 *Apparatus*

(a) *Graham condenser.*—No substitutes, with $^{24}/_{40}$ joints, jacket length 200 mm (Kontes No. K-439000).

(b) *Kuderna-Danish (K-D) evaporative concentrator.*—250 mL capacity, with $^{24}/_{40}$ column connection and $^{19}/_{22}$ lower joint, complete with springs (Kontes No. K-570000).

(c) *K-D concentrator tube.*—4 mL capacity, with $^{19}/_{22}$ joint, and 0.1 mL subdivisions from 0 to 2.0 mL (Kontes No. K-570050). Check accuracy of graduations. Use with pennyhead stoppers ($^{19}/_{22}$ joint).

(d) *Snyder column.*—3 section, 150 mm, with $^{24}/_{40}$ joints (Kontes No. K-503000).

(e) *Micro Snyder column.*—3 chambers, with $^{19}/_{22}$ joint (Kontes No. 569001:3-19).

(f) *GLC columns.*—6 ft × 1/8 in. (od) stainless steel column packed with 20% Carbowax 20M and 2% NaOH on 80–100 mesh acid-washed Chromosorb P. Column must be able to handle 10 μL sample ext and must give good resolution of NDMA peak from both solv. (in beer ext) and NDPA peaks. Injector and column temps, 220° and 170°, resp. Carrier gas (Ar) flow, 25–30 mL/min.

(g) *GLC-thermal energy analyzer.*—Thermo Electron Corp., Waltham, MA, connected to 1 mV recorder. Operate according to instrument manual and with −110° to −130° slush bath. Adjust instrumental parameters, such as vac. chamber pressure, O flow, calibration knob, etc., to obtain proper sensitivity. Set recorder chart speed at ca 0.5 cm/min.

Note: Thoroly clean all glassware before use. After normal cleaning and washing, wash with chromic acid. If contamination still exists, rinse all glassware with CH_2Cl_2 before use. Let charred residue in distn flask soak with dil. alkali and then wash in normal manner.

10.C13 *Sampling and Storage*

Store beer sample at 4° in dark and analyze as soon as possible. When opening bottle or can, transfer ca 120 mL aliquot into g-s erlenmeyer and store as above. Alternatively, recap bottle, using bottle capper, after taking aliquot. In the latter case, test 4% alc. ext of new cap liner for NDMA contamination before use.

10.C14 *Distillation*

Accurately weigh 50 ± 0.1 g beer into 1 L distn flask and add 1.0 mL each of 10% sulfamic acid, NDPA internal std (250 ng/mL), and 1N HCl. Mix contents by gentle swirling and let stand in dark 10 min. Then add 10.0 mL 3N KOH and 2 small Boileezers, and mix. Set up distn app. so that connecting adapter slopes downward toward vertical Graham condenser. Loosely wrap glass wool around distn flask and connecting adapter. Set up 100 mL graduate under condenser to collect distillate. Cooling H_2O for condenser should be ≤20°.

During initial 10 min of distn, adjust rheostat (usually at 50) so that mixt. boils smoothly without too much frothing or bumping. Watch constantly for excessive foaming and, if necessary, turn off heat for 1–2 min. After 10 min, increase rheostat setting to 60 or 65 and continue distn (watch for foaming) until most of soln is distd. Stop distn when ca 55 mL distillate is

collected. *Do not heat distg flask to complete dryness;* this may give erroneous results. Total distn time should be ≤1 h. If any portion of sample foams over during distn, discard experiment and start over with fresh aliquot.

10.C15 *Extraction and Cleanup*

Disconnect adapter after distn; do not rinse adapter. Add 2.0 mL 10N KOH to distillate in graduate and transfer to 250 mL separator. Use same cylinder for all subsequent measuring of CH_2Cl_2. Rinse condenser with 50 mL CH_2Cl_2, and collect rinsing directly into separator contg distillate and KOH. Ext distillate with CH_2Cl_2 by shaking vigorously 2 min and drain off CH_2Cl_2 layer into second separator. Ext aq. layer with 2 addnl 50 mL portions of CH_2Cl_2 and combine all CH_2Cl_2 exts in second separator. Discard aq. layer.

Place 40 g anhyd. Na_2SO_4 in coarse sintered-glass buchner, wash with ca 20 mL CH_2Cl_2, and discard washing. Assemble 250 mL K-D evaporative concentrator with 4 mL concentrator tube at bottom. While connecting bottom tube, wet joint with CH_2Cl_2 and attach springs. Dry combined CH_2Cl_2 ext by passing through Na_2SO_4 bed on buchner and collecting ext directly in K-D concentrator. Wash Na_2SO_4 bed with further 20 mL CH_2Cl_2 and collect washing in K-D concentrator.

10.C16 *Concentration*

Add 1 tiny piece (1-2 mm) of Boileezer to contents of K-D flask, attach 3-section Snyder column, and conc. ext by heating flask in H_2O bath (50-60°). Initially maintain outside H_2O level close to level of CH_2Cl_2 inside flask and continue heating until concd ext is ca 4 mL (ca 40 min). (If excessive boiling occurs during concn, control it either by raising flask slightly out of H_2O bath or by decreasing temp. of bath.) Finally raise flask above H_2O and let condensed CH_2Cl_2 in Snyder column drain into flask. Add ca 1 mL CH_2Cl_2 to top of Snyder column and let it drain into flask. Disconnect concentrator tube from flask.

Add another tiny piece of Boileezer to contents and attach micro Snyder column and springs. Conc. ext to ca 0.8 mL by heating concentrator tube in 50-60° H_2O bath. Lift out or immerse tube in H_2O to control boiling rate but *do not* lift tube completely out of H_2O bath; this will stop action of Boileezer. *Avoid overheating and excessive accumulation of CH_2Cl_2 in column chambers.* Stop concn when CH_2Cl_2 level reaches ca 0.8 mL; do not conc. to less than 0.8 mL. Carry out this final concn step slowly, taking at least 30 min. Raise tube (bottom still touching H_2O), let liq. drain, and note vol. to see if it is ca 0.8 mL. If >0.8 mL, continue concn as above. Finally, rinse micro Snyder column with a few drops of CH_2Cl_2, let rinsing drain to tube, disconnect column, and dil. ext to 1.0 or 1.1 mL (not >1.1 mL). (Do not use N stream for concentrating ext at any stage.)

Stopper tube, mix in vortex mixer, and store at 4° in dark until analysis. Let warm to room temp. and note vol. before analyzing ext.

10.C17 *Reagent Blank*

To ensure absence of contamination, carry out reagent blank taken thru all steps as mentioned above, except use 50 mL 4% alcohol in H_2O instead of 50 g beer. Inject 10 μL ext for GLC-TEA analysis as described below.

10.C18 *Determination of Standard Curve*

Set attenuation (usually 4) of TEA detector so that injection of 30 pg NDMA gives definite peak with acceptable background. Using this attenuation, analyze 6 μL aliquots, in duplicate, of NDMA stds 5, 10, 20, and 40 ng/mL. Before injection, draw out syringe plunger slightly and note exact vol. of ext to be injected (there must be a small air gap between sample and rinsing solv. already inside needle). During injection, make sure no sample is lost thru back of plunger due to back pressure. After injection, hold needle in septum 5 s before withdrawing.

Next, choose a higher attenuation setting that gives on-scale peak for 6 μL of NDMA std 500 ng/mL. Using this setting, analyze 6 μL aliquots, in duplicate, of NDMA stds 500, 200, 100, and 40 ng/mL.

Accurately measure peak hts (±0.1 cm) and det. av. peak hts of 2 injections at each concn. If exactly 6 μL is not injected, make appropriate corrections and convert all peak hts equiv. to 6.0 μL injections. Draw 2 std curves, one for each attenuation setting, peak hts vs pg injected. Det. std curve weekly.

10.C19 *Analysis of Beer Extract*

As above, inject 6 μL aliquots of beer ext, in duplicate, using lowest attenuation setting sensitive to 30 pg NDMA. Measure and det. av. peak ht corresponding to 6.0 μL injection. Compare this av. peak ht with std curve and det. which std NDMA soln, when injected under same attenuation setting, produces closest peak ht. Choose that NDMA std soln, inject 6 μL aliquots, in duplicate, and det. av. peak ht.

If sample ext on first injection produces off-scale peak, choose a higher attenuation setting (16 or 32) and carry out analysis, in duplicate, as above. Also analyze corresponding std NDMA soln at same attenuation. For samples giving off-scale peaks at attenuation 32, dil. exts with CH_2Cl_2 to 5.0 mL in a vol. flask and re-analyze. *For accurate results, analyze beer ext and corresponding std under same attenuation setting and all within 60 min.*

If, on the other hand, ext gives neg. result for NDMA or peak is too small to measure, inject 10 μL aliquots, in duplicate (use 25 μL syringe). Similarly, inject duplicate 10 μL aliquots of NDMA std 5 ng/mL for quantitation. To achieve 0.1 ppb detection limit, 10 μL aliquots of beer ext must be analyzed under attenuation setting that gives detectable peak for 30 pg NDMA.

Note: If using 25 μL syringe, which usually has thick needle, watch for septum damage and check for leaks. To be on safe side, *use a new septum* daily.

20.C20 *Calculation*

Calc. concn of NDMA in beer, using following formula:

$$\text{Uncorrected ppb NDMA in beer} = (h_1 p v_2)/(h_2 g v_1)$$

where h_1 = av. NDMA peak ht (cm) of beer; h_2 = av. peak height (cm) of corresponding NDMA std; p = pg NDMA that produced h_2 peak ht; v_1 = μL beer ext injected; v_2 = final vol. (mL) of beer ext; g = g beer taken for analysis.

Correction for % recovery of NDPA.—Accurately measure peak ht of NDPA peak on each beer chromatogram and calc. av. peak ht of 2 injections. Make appropriate corrections if final vol. of beer ext is not exactly 1.0 mL or injection vol. is not exactly 6.0 μL. Then (within 60 min) inject, in duplicate, 6 μL NDPA std (250 ng/mL) under same attenuation setting. Calc av. peak ht and correct value if exactly 6.0 μL is not injected. Calc. % recovery of NDPA for each sample. If recovery of NDPA is less than 80%, repeat analysis from beginning. Finally, correct results as follows:

Corrected ppb NDMA in beer = (uncorrected ppb/% recovery of NDPA) × 100.

---

(4) The following method for the determination of barley extract, Report of Subcommittee on Methods of Barley Analysis (May 1, 1945) American Society of Brewing Chemists, 3340 Pilot Knob Rd, St. Paul, MN, p. 31; Report of Subcommittee on Methods of Barley Analysis (1946) *Am. Soc. Brew. Chem. Proc.*, p. 92; *J. Assoc. Off. Anal. Chem.* **64**, 1138 (1981), was adopted official first action as an ASBC–AOAC method:

Collaborative Study

Ten bottles of beer consisting of 5 pairs of blind duplicates were sent to each of 13 laboratories. They were asked to carry out single analyses on each sample and report the results on the forms provided. Samples consisted of the following beers: Nos. 1 and 6 were a commercial beer taken from the same batch; Nos. 2 and 7 were a specially made commercial beer extremely low in NDMA content (made from a low NDMA malt); Nos. 3 and 10 were the low NDMA beer spiked with 0.5 ppb NDMA; Nos. 4 and 12 were the low NDMA beer spiked with 1.9 ppb NDMA; and Nos. 5 and 9 were the low NDMA beer spiked with 5.0 ppb NDMA. To obtain information on day-to-day variation of results for duplicate samples, laboratories were requested to follow a predetermined schedule of analysis so that no 2 replicates would be analyzed on the same day. They were also asked, for the reason explained below, to weigh the content (±1 g) of each bottle and report it with the results. Each laboratory was instructed to procure and prepare its own standards of NDMA and *N*-nitrosodi-*n*-propylamine (NDPA), used as an internal standard. Thus, only the samples and a copy of the method were provided to the laboratories.

Because no appropriate facilities to prepare large batches of spiked beers were available at the Associate Referee's laboratory, and several breweries contacted were unable to prepare them in their plants, the preparation of these samples presented a special problem. The only choice left was to spike each bottle individually. For the spiking experiment, a special blank beer was made (through the courtesy of Carling O'Keefe Breweries of Canada Ltd, Toronto, Ontario) from a malt that was extremely low in NDMA content. Each bottle of this beer, which had an average NDMA content of 0.1 ppb, was filled by using a manually operated filler so that the variation of contents from bottle to bottle would be minimum. Subsequent measurements of the contents of 8 randomly selected bottles were 342.2 ± 0.1 mL (SD), ensuring their uniformity in contents. Moreover, each collaborator also measured the content of each bottle, thus elimi-

nating any possibility of error in filling. Each bottle was carefully spiked with 5.0 mL of diluted NDMA standards of appropriate strength, recapped immediately, mixed well, and stored at 4°C until ready to be shipped.

To prevent breakage and to expedite delivery, samples were sent by air freight in specially designed styrofoam containers. All laboratories except one received them within 2–3 days. Because of delay in customs, one laboratory did not receive the samples until 2–3 weeks later. There was just enough space for 10 bottles in the specially designed container, so no practice sample was sent. Instead, each laboratory was asked to analyze Sample 1 first, to report the results by telephone or telex within 1 week, and to proceed with the analysis of the rest of the samples only after being advised by the Associate Referee to proceed. They were informed beforehand that this was not a practice sample and the result they reported would be final. This arrangement worked well, and all except one laboratory obtained the expected results on first analysis. Collaborators were asked to complete the analysis of the rest of the samples within 2 weeks of completing the first step.

Statistical Methods

The study yielded a set of data, x_{ijk}, where i denotes the laboratory, j denotes the level of contamination, and k denotes the individual sample within a level. The analysis consisted of 3 parts: identification of outliers, estimation of variance components, and estimation of percent recovery for the overall method. Analysis was done on both the reported data and on the natural logarithms of the data. (This latter analysis, which effectively considers relative rather than absolute errors, would be the appropriate one if the coefficient of variation were constant across all levels.)

Tests for outliers were done on the means of duplicates $(x_{ij1} + x_{ij2})/2)$, on differences between the duplicates $(x_{ij1} - x_{ij2})$, and on the overall results of the laboratory, by using methods of Cochran (6), Dixon (7), and Thompson and Wilke (8) as described by Steiner (9).

Within each NDMA level, variance components of between-laboratory (σ_L^2) and within-laboratory (σ_E^2) variation, and repeatability and reproducibility were calculated as described by Steiner (9). In addition, following Steiner, an analysis combining all levels was performed, but this joint analysis was done on the natural logarithms of the data. The joint analysis gives 2 components of variance in addition to the within-laboratory error. These are a laboratory effect (σ_L^2) which quantifies the differences between laboratories that are consistent across all levels, and a laboratory–sample interaction (σ_{LS}^2) which quantifies laboratory effects specific to certain levels. (Because the analysis is done on the log scale, a "consistent" difference is defined as a proportionate one, e.g., a laboratory which overestimates a 1 ppb sample as 1.1 ppb will also overestimate a 5 ppb sample as 5.5 ppb.) Overall reproducibility was calculated as $\sqrt{\sigma_L^2 + \sigma_{LS}^2 + \sigma_E^2}$. Standard deviations of logarithmically transformed data can be interpreted as coefficients of variation, so these estimates were not divided by the mean.

These analyses follow standard random effects methods (10, 11) with samples nested within laboratories (random effects) and for the joint analysis, NDMA levels (fixed effects) crossed with laboratories.

Because 8 of the 10 samples were in fact similar samples spiked with known amounts of NDMA, it is possible to estimate the bias. If one assumes that each laboratory has a bias proportional to the amount present (i.e., constant percent recovery), then the bias can be modeled by

$$y = \beta(z + x)$$

where z is the amount naturally present, x is the amount added, and y is the amount reported. The coefficient, β, measures the percent recovery. The parameters β and z were estimated for each laboratory by a method as described in the appendix.

Results and Discussion

Table 1 is a compilation of results reported by the participating laboratories. The percentage recoveries of NDPA internal standard for all analyses are given in Table 2. As can be seen, except in the case of Laboratory B the percentage recoveries were excellent. All laboratories were also able to detect the low level of NDMA in Samples 2 and 7, thus confirming the minimum detection limit of the method which had been claimed to be 0.1 ppb.

As mentioned above, all laboratories weighed the content of each bottle. Their data confirmed our findings that the bottles were uniformly filled. The average (± SD) for all unspiked, low NDMA beers (Nos. 2 and 7) and those (Nos. 3, 4, 5, 9, 10, 12) spiked with 5 mL NDMA standard solution (in 10% ethanol) were 341.5 ± 1.7 g and 346.5 ± 2.2 g, respectively. By checking the content of each bottle, it was possible to detect a spiking error for Sample 5 for Laboratory K,

Table 1. Results reported by various laboratories: ppb NDMA detected [a]

Sample [b]	A	B	C	D	E	F	G	H	I	J	K	L	M	Mean [c]
1	0.6	0.5	0.6	0.5	0.6	0.6	0.5	0.7	0.4	0.8	0.5	0.6	0.4	0.58
6	0.6	0.6	0.5	0.4	0.6	0.6	0.5	0.5	0.5	0.4	0.6	0.7	0.4	0.54
2 [d]	0.10	0.23	0.13	0.11	0.15	0.11	0.12	0.12	0.13	0.16	0.13	0.13	0.1	0.13
7	0.12	0.28	0.18	0.11	0.11	0.10	0.20	0.17	0.13	0.11	0.16	0.11	0.1	0.16
3	0.7	0.8	0.7	0.5	0.7	0.7	0.7	0.7	0.5	0.6	0.7	0.6	0.4	0.66
10	0.6	0.9	0.6	0.5	0.7	0.6	0.7	0.7	0.5	0.6	0.7	0.7	0.1	0.65
4	2.1	2.0	2.0	1.9	2.2	2.1	2.3	2.2	1.7	1.7	1.9	2.4	1.1	2.04
12	1.9	2.3	2.2	1.9	2.1	2.2	2.1	2.3	1.8	1.7	2.1	2.6	1.2	2.10
5	5.9	5.2	5.0	4.8	5.6	5.6	5.9	5.2	4.5	3.7	5.0	6.1	2.9	5.20
9	5.1	5.2	5.1	5.0	5.3	5.7	5.7	5.9	4.4	4.0	5.1	6.3	2.9	5.23

[a] All results corrected for % NDPA recoveries.

[b] Identity of the duplicate samples: 1 and 6 = commercial beer; 2 and 7 = commercial beer made from a malt low in NDMA; 3 and 10 = low NDMA beer spiked with 0.5 ppb NDMA; 4 and 12 = low NDMA beer spiked with 1.9 ppb NDMA; 5 and 9 = low NDMA beer spiked with 5 ppb NDMA.

[c] Excluding Laboratory M.

[d] Values were recalculated from the raw data.

which reported a level of 10 ppb NDMA (instead of 5.0 ppb) for this sample. On examining the raw data supplied by this laboratory, it was found that Sample No. 5 contained 352.1 g beer whereas the corresponding duplicate (No. 9) as well as the other spiked beers all contained between 346.7 and 347.6 g. The difference amounted to 5 g which must have resulted from spiking the bottle twice with 5.0 mL aliquots of standard NDMA solution. The reported result for this sample was eventually corrected to 5.0 ppb.

Most laboratories followed the procedure as described, but a few laboratories made some minor changes. For example, Laboratory H used an Extreleut column (instead of a separatory funnel) for extracting the distillate, and Laboratories B and J used different concentrations of NDMA and/or NDPA than called for in the method for quantitation of NDMA level and for calculating percent recoveries of added NDPA. Laboratories H and L used electronic integrators for quantitation of peak areas whereas all others measured peak heights as suggested in the method. One laboratory (J) changed the GLC column (from FFAP to Carbowax 20M + KOH) and readjusted TEA operating parameters after analyzing Sample 1. These changes improved the sensitivity by 100%.

For every sample, Laboratory M (delayed samples) reported values lower than those from any other laboratory. The deficiency was considerable in the higher level samples. Laboratory M could thus be considered an outlying laboratory on the basis of the Thompson and Wilke (8) test. Laboratory M was omitted before we checked for other outliers.

Dixon's test (7) on the means of the duplicates indicated that Laboratory B had atypically high values for the low NDMA beers (Nos. 2 and 7). Analysis on the log scale confirmed this outlier, and also pointed to Laboratory J as having atypically low levels in the 5 ppb spiked samples (Nos. 5 and 9). Failure to use the exact quantitation procedure for measuring the levels of NDMA (Laboratory J) and poor chromatograms (tailing solvent peak) in the case of Laboratory B may have been the cause of these discrepancies. It is difficult to measure peak heights accurately

Table 2. Percentage recoveries of NDPA internal standard in different samples

Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	Av. [a]
1	94.9	85.0	94.5	101.3	95.2	101.1	84.7	84.8	95.0	105.7	92.0	90.0	97.4	94.0
6	101.0	67.0	95.7	98.6	95.6	83.7	83.3	76.7	102.1	97.8	100.8	99.0	96.6	94.2
2	102.5	66.0	90.0	91.5	96.8	92.9	85.5	86.0	93.3	107.9	99.1	111.0	85.9	95.2
7	92.6	75.0	98.2	91.4	99.1	96.9	84.8	88.6	107.7	98.4	95.4	109.0	91.2	96.1
3	103.8	63.0	99.9	98.1	98.5	95.7	78.7	77.6	103.2	109.6	97.8	102.0	91.2	96.3
10	95.5	76.0	96.5	95.7	99.9	95.9	87.2	86.1	91.9	87.4	89.6	86.0	91.3	91.9
4	95.0	69.0	96.7	98.1	96.6	98.9	78.8	84.3	100.7	107.0	94.0	91.0	94.6	94.6
12	106.0	61.0	97.4	97.5	102.5	102.0	90.5	82.0	99.0	85.0	93.4	95.0	84.5	94.6
5	86.7	76.0	101.0	99.5	95.0	90.8	81.1	80.7	105.6	96.9	92.0	98.0	100.0	93.9
9	103.0	74.0	95.7	98.9	98.7	75.9	86.0	85.6	103.4	89.5	90.0	93.0	92.3	92.7

[a] Excluding Laboratory B.

in a tailing baseline if the peak is too small. This was the case with Samples 2 and 7 which contained 0.1 ppb NDMA, the detection limit of the method.

Dixon's test (7) on the differences of the duplicates indicated that Laboratory J had an atypically large difference between Samples 1 and 6. Cochran's test (6) on the log scale confirmed this outlier, and also picked out Laboratory G as having an atypically large difference between the 2 low NDMA samples (Nos. 2 and 7). As pointed out earlier, Laboratory J changed analytical parameters after analysis of Sample 1, which may or may not have been the cause of the large difference mentioned above. Because of using an improper GLC column different from that suggested, Laboratory G had the same problem of solvent peak tailing as Laboratory B (see above) and had difficulty measuring small peaks (Nos. 2 and 7) on a tailing baseline. Laboratories which used the suggested or similar columns did not have such problems and obtained good reproducibility between duplicates even at low (0.1 ppb) levels.

Examination of the data in Table 2 indicates that Laboratory B consistently obtained lower recoveries for NDPA internal standard than did the other laboratories. This laboratory used one NDPA standard for spiking the samples and a different one, approximately 5 times more concentrated, for comparison by GLC analysis. Because of this difference in strength of the 2 solutions, the 2 GLC analyses (for sample and standard NDPA) were carried out under 2 different attenuation settings (8 and 64), whereas the method clearly stipulated that the NDPA solutions used for spiking and quantitation must be of the same strength and must be analyzed at the same attenuation setting. Although other explanations are possible, this deviation from the method could have been the reason for the low recoveries. As a result, some values might have been over-corrected for percentage recoveries. For the above reason, Laboratory B was considered an outlier in some of the calculations discussed later.

The mean values, components of variation, and measures of repeatability and reproducibility of the complete data are given in Table 3. This table also gives the statistics for the pooled data on a logarithmic scale.

As can be seen from the data in Table 3, the coefficient of within-laboratory variability (repeatability) decreases with increasing level of NDMA. However, such an effect is not so apparent with the reproducibility. All the samples except Nos. 1 and 6 showed significant laboratory effects. On the log scale, both laboratory effects and laboratory–sample interaction are statistically significant. It thus appears that the laboratory effect is not consistently proportional to the level of NDMA.

If, however, one takes out the data from Laboratories B and M and for Sample 1 from Laboratory J as outliers, a much improved picture emerges (Table 4). Reproducibility coefficients of variation are greatly reduced and laboratory–sample interaction is no longer significant. In view of the explanations given above, exclusion of data from Laboratories B and M and for Sample 1 from Laboratory J is justified.

Estimates of percent recovery and blank concentration and their variance components are given in Tables 5 and 6. For all laboratories ex-

Table 3. Repeatability and reproducibility (complete data)

For Different Levels, Original Scale

		Repeatability		Lab. effect	Reproduc bility	
Samples	Mean, ppb	σ_E, ppb	CV, %	σ_L, ppb	$\sqrt{\sigma_L^2 + \sigma_E^2}$, ppb	CV, %
1, 6	0.55	0.10	18	0.224	0.10	19
2, 7	0.142	0.028	19	0.035*	0.45	32
3, 10	0.62	0.07	12	0.13*	0.15	25
4, 12	2.00	0.11	5.7	0.32*	0.34	17
5, 9	5.04	0.24	4.7	0.9*	0.90	18

For Levels Pooled, Log Scale

Repeatability	Lab. sample interaction	Lab. effect	Reprocucibility
σ_E (%)	σ_{LS} (%)	σ_L (%)	$\sqrt{\sigma_E^2 + \sigma_{LS}^2 + \sigma_L^2}$ (%)
17	9.6*	18*	27

* Variance component significantly different from zero ($P < 0.05$).

Table 4. Repeatability and reproducibility after omitting all data from Laboratories B and M and for Sample 1 from Laboratory J

For Different Levels, Original Scale						
		Repeatability		Lab. effect	Reproducibility	
Samples	Mean, ppb	σ_E, ppb	CV, %	σ_L, ppb	$\sqrt{\sigma_L^2 + \sigma_E^2}$, ppb	CV, %
1, 6	0.55	0.07	12	0.06	0.09	15
2, 7	0.13	0.028	22	<0	0.027	21
3, 10	0.64	0.043	6.7	0.07*	0.08	13
4, 12	2.06	0.10	5.1	0.22*	0.24	12
5, 9	5.22	0.26	4.9	0.63*	0.70	13

Across All Levels Pooled, Log Scale			
Repeatability	Lab. sample interaction	Lab. effect	Reproducibility
σ_E (%)	σ_{LS} (%)	σ_L (%)	$\sqrt{\sigma_E^2 + \sigma_{LS}^2 + \sigma_L^2}$ (%)
11	5.8	8.6*	15

* Variance component significantly different from zero ($P < 0.05$).

cept M and J, the relationship between amount spiked and amount reported was linear, thus justifying the use of the model. From data in Table 5 it can be said with 95% confidence that the true percent recovery is between 93.7 and 109.1% (after excluding Laboratory M on the basis of Dixon's test). Thus, there is no evidence that the method is biased. On the basis of data in Table 6, there seems to be significant laboratory-to-laboratory variation in percent recovery, whether Laboratory M is included or not. Thus, although the method is not biased on the average, the percent recovery for a laboratory picked at random could be (with 95% confidence) found in the range of 75–127.8%, even if Laboratory M is excluded. Since each laboratory procured and prepared its own standards, such laboratory-to-laboratory variations could partly be due to variations in the strength of NDMA standards in different laboratories. One might have obtained better results if each laboratory had been provided with the standard nitrosamine solutions but this would not have been realistic; various regulatory laboratories in their daily work usually prepare their own standards. Therefore, results obtained in this study represent the worst possible data one can get under normal working conditions in various laboratories.

There do appear to be laboratory effects on the blank concentration (Table 6) when all laboratories are included. However, these laboratory effects disappear if Laboratory B (see Tables 5, 6)

Table 5. Estimates of percent recovery and blank concentration by laboratory

Lab.	Recovery, %	Blank concn, ppb
A	105.2	0.102
B	99.2	0.275*
C	99.0	0.155
D	94.7	0.102
E	106.6	0.124
F	110.0	0.093
G	111.6	0.131
H	108.6	0.133
I	85.8	0.139
J	76.5	0.194
K	98.3	0.155
L	121.8	0.090
M	54.2*	0.050
Mean ± SE (all labs)	97.8 ± 4.9	0.134 ± 0.105
Omitting Lab. M	101.4 ± 3.5	—
Omitting Lab. B	—	0.122 ± 0.011
Omitting Labs B, J, M	—	0.122 ± 0.008

* Significant outlier ($P < 0.05$) by Dixon's test.

Table 6. Variance components for estimated percent recovery and blank concentration

Item	Repeatability (estimation error) σ_E	Lab. effect σ_L	Reproducibility $\sqrt{\sigma_E^2 + \sigma_L^2}$
Percent recovery (100 $\hat{\beta}$)			
All labs	2.9	17*	17
Excluding Lab. M	2.3	12*	12
Blank concentrat on $\hat{\gamma}$ (ppb)			
All labs.	0.037	0.042*	0.056
Excluding Lab. B	0.037	0.008	0.038
Excluding Labs. B, J, M	0.019	0.016	0.024

* Variance component significantly greater than zero ($P < 0.05$).

is excluded. It thus appears that, except for Laboratory B which seems to have an additive error, the laboratories give consistent estimates for the blank concentration. A very precise (0.122 ± 0.008 ppb) estimate of blank concentration is obtained if Laboratories J and M, which displayed a nonlinear relationship between amount added and amount reported, are also excluded. For a measurement at ≅0.1 ppb level, this can be considered extremely good.

Collaborator Comments

Most laboratories completed the analysis with no difficulty and had no adverse comments. However, 2 laboratories (I and M) commented that the method was somewhat lengthy and unsuitable for rapid screening of a large number of samples. The Associate Referee agrees, but feels that, with 2 distillation setups one analyst should be able to test 4 samples/day. One laboratory (H) reported that the use of strong alkali badly corroded the distillation flask. During GLC analysis, Laboratory B initially found tailing solvent peaks on one TEA apparatus but got satisfactory results for repeat analyses on another TEA apparatus in the same laboratory. The reason for this difference was not apparent.

All collaborators were informed that it might be necessary to use a GLC column of high Carbowax 20M loading (20%) and some NaOH (2%) to obtain good resolution of NDMA peak from solvent peak. Five laboratories (C, E, F, J, K) commented that the use of such a column definitely improved solvent peak tailing and gave better resolution of NDMA peak from the solvent peak (especially in beer extracts). One laboratory (I), on the other hand, disagreed with the need for using NaOH in the GLC column packing. This laboratory, however, used a column that was high (15%) in Carbowax 20M content and obtained excellent chromatograms. The Associate Referee feels that a few laboratories did not take the problem seriously enough and used GLC columns that gave less than satisfactory resolution of NDMA peak from the solvent peak. Those who used columns of high Carbowax loading (15–20%) or of lower (10%) loading but of longer lengths (9–10 ft) obtained excellent chromatograms.

Two laboratories (B, H) reported the presence of an unknown peak between NDMA and NDPA. This, however, did not interfere with the analysis. Laboratory H observed this unknown peak even in reagent blanks. It could have originated from one of the reagents or the Extreleut column which was used by Laboratory H (see earlier) for extraction purposes. No other laboratory reported the presence of such peaks.

Six laboratories (C, D, F, I, J, K) commented that the method worked well and gave excellent recoveries for added NDPA internal standard.

Summary and Conclusions

Analysis of the data (after omitting the outliers) suggests that the overall method gives results that are unbiased (101.4 ± 3.5% recovery), and the pooled coefficients of variation of the data are also excellent (11% for repeatability and 15% for reproducibility). Even when all outlying laboratories are included, the corresponding pooled coefficients of variation (17% and 27%) are highly satisfactory for such low level (0.1–5 ppb) measurements and they are within the acceptable guidelines suggested by Horwitz et al. (12). It should also be noted that coefficients of variation for repeatability include day-to-day variations. One may argue against omitting the outliers, but reasoning behind excluding each outlier has been explained and supported by valid statistical analysis.

It is, therefore, recommended that the method with the changes mentioned earlier be adopted official first action for the determination of NDMA in beer and ale.

Acknowledgments

The Associate Referee thanks the following collaborators for participating in the study: M. Castegnaro, International Agency for Research on Cancer, Lyon, France; G. Ellen, Rijksinstituut voor de volksgezondheid, Bilthoven, The Netherlands; T. Fazio, Food and Drug Administration, Washington, DC; W. Fiddler, Eastern Regional Research Laboratories, USDA, Philadelphia, PA; D. H. Fine, New England Institute for Life Sciences, Waltham, MA; W. C. Herwig, Miller Brewing Co., Milwaukee, WI; P. Issenberg, Eppley Institute for Research in Cancer, University of Nebraska, Omaha, NE; A. B. Lacey, Barrett Burston (Australia) Ltd, Melbourne, Australia; R. Massey, Ministry of Agriculture, Fisheries and Food, Norwich, UK; T. O'Brien, Great Western Malting Co., Vancouver, WA; R. Scanlan, Oregon State University, Corvallis, OR; G. Thomas, Ontario Research Foundation, Toronto, Ontario, Canada; J. Thomsen, Danish Institute of Protein Chemistry, Horsholm, Denmark

The Associate Referee is also grateful to the Brewers Association of Canada and Carling O'Keefe Breweries of Canada Ltd, Toronto, On-

tario, for their assistance in preparing the beer samples used in this study.

REFERENCES

(1) Walker, E. A., Griciute, L., Castegnaro, M., & Börzsönyi, M. (1980) in *N-Nitroso Compounds: Analysis, Formation and Occurrence*, IARC Scientific Publication No. 31, International Agency for Research on Cancer, Lyon, France
(2) Spiegelhalder, B., Eisenbrand, G., & Preussmann, R. (1980) in *N-Nitroso Compounds: Analysis, Formation and Occurrence*, IARC Scientific Publication No. 31, International Agency for Research on Cancer, Lyon, France, pp. 467–477
(3) Sen, N. P. (1981) presented at the AOAC Spring Workshop, May 12–14, 1981, Ottawa, Ontario, Canada
(4) Havery, D. C., Hotchkiss, J. H., & Fazio, T. (1981) *J. Food Sci.* **46**, 501–505
(5) Sen, N. P., & Seaman, S. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 933–938
(6) Cochran, W. G. (1941) *Ann. Eugenics* **11**, 47
(7) Dixon, W. J. (1953) *Biometrics* **9**, 74
(8) Thompson, W. A., & Wilke, T. A. (1963) *Biometrika* **50**, 375
(9) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA, pp. 65–83
(10) Scheffé, H. (1959) *The Analysis of Variance*, Wiley, New York, NY
(11) Searle, S. R. (1971) *Biometrics* **27**, 1–76
(12) Horwitz, W., Kamps, L. R., & Boyer, K. W. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1344–1354

Appendix: Estimates of Percent Recovery

The parameters β and z were estimated for each laboratory by fitting the reported values for all samples except 1 and 6 to the model

$$y = \alpha + \beta x \qquad (1)$$

(where x is the amount spiked and y is the amount reported) by empirically weighted least squares. Because a graphical analysis indicated that the variance was proportional to the mean, the fitting was done using the weights y^{-1}. Because the weights used are random variables, the properties of the estimators may not be the same as those of the least squares estimators with known weight. However, the procedure may be used as an approximation.

Nonlinearity of the relationship was assessed by testing the significance of the parameters γ and δ in the models.

$$y = \alpha + \beta x + \gamma x^2$$

$$y = \alpha + \beta x + \gamma x^2 + \delta x^3 \qquad (2)$$

respectively, using the between duplicate error term (with 4 degrees of freedom). These tests of nonlinearity were done separately for each laboratory, using the empirical weights y^{-1}.

From the estimates $\hat{\alpha}$ and $\hat{\beta}$ of equation (*1*), the parameter z can be estimated by $\hat{z} = \hat{\alpha}/\hat{\beta}$, and the variance of $\hat{z}$ can be approximated by

$$\text{Var } \hat{z} = 1/\hat{\beta}^2 [\text{Var } (\hat{\alpha}) + \hat{z}^2 \text{ Var } (\hat{\beta}) - 2\,\hat{z}\,\text{Cov } (\hat{\alpha}, \hat{\beta})] \qquad (3)$$

Let β_i be the true recovery rate of Laboratory i, then $e_i = \hat{\beta}_i - \beta_i$ is the estimation error of the parameter, and we can write

$$\hat{\beta}_i = \beta_i + e_i \qquad (4)$$

Assume that the laboratories can be considered a random sample of some population, and that $\overline{\beta}$ is the true (average, unconditional) recovery. Then if we assume that the estimators are unbiased, with errors uncorrelated with the true values (these assumptions are only approximately true because the empirical weighting leads to nonlinear estimates), we can say

$$E(\hat{\beta}_i) = E(E(\hat{\beta}_i | \text{Lab. i})) = E(\beta_i) = \overline{\beta}$$

$$\text{Var } (\hat{\beta}_i) = E(\text{Var } (\hat{\beta}_i | \text{Lab. i})) + \text{Var } (E(\hat{\beta}_i | \text{Lab. i}))$$

$$= \sigma_E^2 + \sigma_L^2 \qquad (5)$$

$\overline{\beta}$ and Var $(\hat{\beta}_i)$ were estimated in the obvious way, by the mean and variance across laboratories σ_E^2 was estimated by the average of the variances obtained from the least square estimates within each laboratory and σ_L^2 was obtained by subtraction. The hypothesis $\sigma_L^2 = 0$ was tested by the F-ratio $\hat{\text{Var}}(\hat{\beta}_i)/\hat{\sigma}_E^2$.

A similar analysis was done for the parameter z, estimating σ_E^2 by averaging (*3*) across laboratories.

Evaluation of Thin Layer, Paper, and High Performance Liquid Chromatography for Identification of Dyes Extracted as Ion-Pairs with Tri-*n*-octylamine

MARC L. PUTTEMANS, LOUIS DRYON, and DÉSIRÉ L. MASSART
Vrije Universiteit Brussel, Farmaceutisch Instituut, Laarbeeklaan 103, B-1090 Brussels, Belgium

Food dyes, extracted into chloroform as ion-pairs with tri-*n*-octylamine, are chromatographed directly in various liquid chromatographic systems. Dyes can be identified by comparison of the chromatographic behavior of ion-pairs with behavior of reference dyes dissolved in water. In thin layer and paper chromatography, the ion-pairs dissociate when polar eluants are used; thin layer chromatography is preferred because of less tailing. In high performance liquid chromatography, a reverse phase ion-pair system is used with tetrabutylammonium as the counter ion; chloroform-extracted dyes show retention times similar to reference dyes dissolved in water.

In the analysis of dyes in food, there are 4 important steps: First, for nonliquid foods, the dyes must be solubilized. Then the colorants must be isolated, identified, and finally quantitated. One of the isolation techniques we reported in an earlier publication (1) is ion-pair extraction, in which an ionized solute is extracted into an organic phase after addition of a suitable counter ion. The counter ion, a hydrophobic ion of opposite charge, forms a hydrophobic complex with the solute which is then extracted to a higher degree than the solute itself by the organic phase. The organic solvent is usually chloroform because of its ability to solvate the ion-pair formed (2).

Acid water-soluble food dyes may be extracted as their ion-pairs with quaternary ammonium ions (3–8) or amines (9). In a previous paper we described the applicability of the counter ion tri-*n*-octylamine (TnOA) in the extraction of acid dyes (9).

The identification techniques used for dyes are usually paper chromatography (PC), thin layer chromatography (TLC), and high performance liquid chromatography (HPLC) (1). Ion-pair extraction of dyes has been described by several authors but all of them had problems in the subsequent chromatography (paper or thin layer) of the extracted dyes. Indeed, the simplest way of identifying the dyes would be to compare the chromatographic behavior of the unknown dye, extracted as its ion-pair, with the behavior of reference dyes, dissolved in water. However, other techniques had to be used in the past. Drevon and Laur (3) and Brustier et al. (4) used reference dyes which had been extracted previously as ion-pairs. Others back-extracted the dyes to an aqueous phase with Teepol (5), Cupferron (6), or HCl (7). Still another technique consisted of applying the ion-pair directly on a paper sheet which had been previously impregnated with an aqueous solution of a sodium or potassium alkyl- or arylsulfonate (8).

Although we observed that dyes extracted with TnOA can be back-extracted into an aqueous phase with HCl, perchloric acid, and in some cases NaOH (9), or by solutions of sodium chloride, bromide, iodide, nitrate, or perchlorate (10), the aim of this article is to demonstrate that it is possible to identify dyes, extracted as ion-pairs with TnOA, by direct chromatographic comparison with aqueous reference solutions. This constitutes an important simplification of the procedure. The possibilities of thin layer, paper, and high performance liquid chromatography are discussed.

Experimental

Ten mL of a solution containing 10 mg dye dissolved in 100 mL phosphate buffer (pH = 5.5, ionic strength = 0.1) was shaken 30 min with 10 mL 0.1M solution of tri-*n*-octylamine (Aldrich-Europe, Beerse, Belgium) in chloroform and then centrifuged. Five mL of the chloroform layer was pipetted into a second centrifuge tube, the chloroform was evaporated, and the residue was dissolved in 10 mL ethanol. Back-extractions were performed by shaking 5 mL chloroform-TnOA-dye solution with 5 mL 0.1M sodium salt solution. Chloroform extracts were used in TLC and PC; the sodium salt and the TnOA extracts, redissolved in ethanol, were used in HPLC.

Thin Layer and Paper Chromatography

Apparatus and Reagents

(a) *Materials*—Silica gel (PN5721) and cellulose (PN5716) precoated plates, 20 × 20 cm

Received July 15, 1981. Accepted November 20, 1981.

0004-5756/82/6503-0730–07$01.00

(Merck); paper sheets (Whatman No. 1), 30 × 12 cm.

(b) *Dyes.*—P. Entrop (Machelen, Belgium) ≥85% pure, used as received.

(c) *Buffers.*—Ionic strength 0.1, prepared from analytical grade sodium phosphate (Merck) and double- distilled water. For 2 L buffer solution, the following quantities are required: pH = 5.50: 1.26 g $Na_2HPO_4 \cdot 2H_2O$ and 24.65 g $NaH_2PO_4 \cdot H_2O$; pH 7.00: 9.38 g $Na_2HPO_4 \cdot 2H_2O$ and 5.77 g $NaH_2PO_4 \cdot H_2O$. pH was adjusted to 5.50 ± 0.05 or 7.00 ± 0.05, if necessary, with 0.1M NaOH, analytical grade (Merck).

(d) *Mobile phases.*—Prepared with pro analysi Merck reagents except for propylamine (Puriss, Fluka AG).

Chromatographic Technique

Eluants were always prepared fresh. Chromatographic tanks were equilibrated 1 h. A volume of 5 or 10 μL of each dye solution (10 mg in 100 mL water) or chloroform extract was spotted on a paper sheet or thin layer plate with a Camag micropipet. Chromatograms were run 15 cm by ascending technique; each R_f value is the average of 2 runs.

High Performance Liquid Chromatography

Apparatus and Reagents

(a) *Liquid chromatograph.*—Varian 8500 with Valco loop injector (10 μL loop) and Varian 254-280 ultraviolet dual beam recording differential photometer with cell volume of 8 μL and cell length of 10 μm; Varian 9176 recorder.

(b) *Column.*—300 mm × 2 mm id MicroPak MCH-10 column packed with 10 μm octadecyl silica.

(c) *Mobile phases.*—5 mL tetrabutylammonium hydroxide (TBA) (25% in methanol, Fluka AG) was diluted to 1 L with one of 2 mixtures of methanol-phosphate buffer (pH 7.00 ± 0.05, ionic strength 0.1): phase A: 30 + 70; phase B: 60 + 40. Methanol was HPLC grade (Fluka); phosphate buffer was prepared from sodium phosphate (pro analysi, Merck). Mobile phases were prepared by mixing appropriate amounts of phases A and B and were always degassed before use.

Procedure

Dyes were dissolved in double-distilled water (10 mg in 100 mL). Each dye and chloroform extract (dried and redissolved in ethanol) was injected twice, recorded at 254 nm, and averaged. The flow was 60 mL/h.

Determination of Tartrazine in Pickles

Five g pickles was macerated in 100 mL 0.1% ammonia solution 1 h at 50°C. The solution was filtered and the pH of the filtrate was adjusted to 5.5 with 0.1% phosphoric acid. The dyes were extracted with 10 mL 0.1M TnOA in chloroform, a 5 mL aliquot of the chloroform phase was evaporated, and the extract was redissolved in 5 mL ethanol. The dye was determined chromatographically as described above.

Results and Discussion

Thin Layer Chromatography

The list of dyes studied is given in Table 1. Schmidt (11) stated that, in general, chloroform–butanol mixtures are suitable eluants for silica gel TLC of salts of amines and organic acids. We tried different butanol–chloroform ratios (Table 2) but found it difficult to separate complex mixtures in one particular system.

A ratio of butanol–chloroform (1 + 8) was chosen, and an acid, acetic acid, and a base, propylamine, were added. The influence of these additions on the R_f values of both the free dye and its TnOA ion-pair are given in Table 3. Of the 5 test dyes selected, 3 show a significant difference between R_f values of the ion-pair and free dye.

For Patent Blue V and Brilliant Green, the similarity of R_f values of the free dye and its ion-pair can be explained by the dissociation of the ion-pairs. The influence of acid is negligible for the free dyes, except Erythrosine. The R_f value of this dye increases from 0.16 to 0.76. The increase of the R_f value of the ion-pairs of Sunset

Table 1. List of dyes studied [a]

Name	EEC No.	CI No.	FDA name
Tartrazine	102	19140	FD&C Yellow No. 5
Quinoline Yellow	104	47005	FD&C Yellow No. 10
Sunset Yellow	110	15985	FD&C Yellow No. 6
Orange GGN	111	15980	
Azorubine	122	14720	Ext. D&C Red No. 10
Amaranth	123	16185	FD&C Red No. 2
Cochenille Red = Ponceau 4 R	124	16255	
Erythrosine	127	45430	FD&C Red No. 3
Patent Blue V	131	42051	
Indigotine	132	73015	FD&C Blue No. 2
Brilliant Green	142	44090	
Brilliant Black	151	28440	

[a] *Colorants for Cosmetics* (1968) 3rd Ed., 1st Rev. (1971) Franz Steiner Verlag, Wiesbaden, GFR.

Table 2. Thin layer chromatography of TnOA–dye ion-pairs on silica gel with butanol–chloroform eluants, and influence of eluant composition on R_f values

	Ratio: butanol–chloroform						
Name	1 + 1	1 + 5	1 + 6	1 + 7	1 + 8	1 + 9	1 + 10
Tartrazine	1.00	0.88	0.12	0.09	0.07	0.07	0.08
Quinoline Yellow	1.00	0.89	0.82	0.06	0.15	0.17	0.76
Sunset Yellow	1.00	0.89	0.84	0.80	0.79	0.71	0.78
Azorubine	0.99	0.88	0.84	0.80t[a]	0.79	0.72	0.76
Amaranth	0.98	0.89	0.84	0.79	0.15	0.72	0.75
Cochenille Red	0.97	0.11	0.22	0.19	0.09	0.07	0.08
Erythrosine	0.97	0.90	0.83	0.79	0.77	0.71	0.72
Patent Blue V	0.10	0.06	0.08	0.07	0.07	0.07	0.06
Indigotine	0.98	0.90	0.16	0.12	0.07	0.07	0.05
Brilliant Green	0.10	0.06	0.07	0.07	0.06	0.07	0.06
Brilliant Black	0.98	0.11	0.28	0.13	0.08	0.07	0.07

[a] Tailing.

Yellow, Azorubine, and Erythrosine with 1% acetic acid is probably due to the addition of a small amount of water with the acetic acid (this reagent has a purity of 96%). This small amount of water is adsorbed by the silica stationary phase and changes its polarity and adsorption capacity.

The addition of propylamine has a dramatic effect on the migration of the free dyes. In an alkaline medium, the dyes are even less soluble in the mobile phase and consequently have an R_f value of zero. The dramatic effect of the addition of propylamine on the ion-pairs is due to dissociation. Erythrosine, which is a rather hydrophobic dye, is still quite soluble in the mobile phase containing 0.1% propylamine but with 0.5% propylamine its R_f is zero. The Erythrosine ion-pair is more difficult to dissociate than other dyes.

To investigate the influence of the polarity of the mobile phase on the migration of the free dye and on the dissociation of its ion-pair, chloroform–alcohol (with varying chain length) (1 + 1) mixtures were tested. Influence of the chain length of the alcohol on the R_f is listed in Table 4. It shows that for the first 3 test dyes the R_f values of the ion-pair and of the corresponding free dye were more similar with increasing polarity of the eluant (decreasing chain length). For Patent Blue V and Brilliant Green, the R_f values are similar with each of the eluants. Therefore, the development of a thin layer chromatographic system where free dyes and dyes spotted as their ion-pairs show identical R_f values, due to the dissociation of the ion-pair, depends on the choice of a polar mobile phase with alkaline pH.

Such TLC systems, for free dyes, are described

Table 3. Thin layer chromatography of dyes (free) and their TnOA ion-pairs (IP) on silica gel with butanol–chloroform (1 + 8) eluant, and influence of acetic acid and propylamine on R_f values

	0%		0.1%		0.5%		1%	
Name	IP	Free	IP	Free	P	Free	IP	Free
				Acetic Acid				
Sunset Yellow	0.84	0.07	0.82	0.07	0.81	0.07	0.91	0.08
Azorubine	0.79	0.07	0.75	0.07	0.78	0.07	0.89	0.08
Erythrosine	0.79	0.16	0.74	0.40	0.76	0.62	0.89	0.76
Patent Blue V	0.07	0.08	0.07	0.07	0.08	0.07	0.08	0.08
Brilliant Green	0.06	0.07	0.07	0.06	0.08	0.07	0.08	0.08
				Propylamine				
Sunset Yellow	0.84	0.07	0.00	0.00	0.00	0.00		
Azorubine	0.79	0.07	0.00	0.00	0.00	0.00		
Erythrosine	0.79	0.16	0.20	0.11	0.14	0.00		
Patent Blue V	0.07	0.08	0.00	0.00	0.00	0.00		
Brilliant Green	0.06	0.07	0.00	0.00	0.00	0.00		

Table 4. Thin layer chromatography of dyes (free) and their TnOA ion-pairs (IP) on silica gel with alcohol–chloroform (1 + 1) eluants, and influence of chain length of alcohol

	Ethanol		1-Propanol		1-Butanol		1-Pentanol	
Name	IP	Free	IP	Free	IP	Free	IP	Free
Sunset Yellow	0.99	0.63	0.99	0.22	1.00	0.20	0.99	0.21
Azorubine	0.99	0.78	0.99	0.26	0.98	0.21	0.99	0.19
Erythrosine	0.99	0.96	0.99	0.97	0.99	0.95	0.99	0.95
Patent Blue V	0.86	0.81	0.12	0.21	0.10	0.10	0.04	0.00
Brilliant Green	0.94	0.72	0.14	0.17	0.10	0.09	0.05	0.00

in the literature for use with cellulose layers (12). Dyes and TnOA ion-pairs were chromatographed in 2 of these systems; the results are given in Table 5. The R_f values of free dyes and their ion-pairs are quite similar. To ensure that the cellulose layer does not cause the dissociation of the ion-pairs, the same mobile phases were also tried with a silica layer. The results, which are shown in Table 6, confirm that dissociation is caused by the polar mobile phases, not some interaction with the chromatographic support.

It is useful to note that silica systems have a lower selectivity than cellulose. Since silica dissolves in alkaline media, the ammonia content of the mobile phase was decreased to increase the stability of the stationary phase. According to the results listed in Table 7, the ammonia content of the mobile phase is not a critical factor in the dissociation of ion-pairs. However, when no ammonia is present, nearly all dyes show tailing spots. In addition to a better stability of the silica layer, the low ammonia content of the mobile phase allows a better interpretation of the chromatogram because some dyes, e.g., Indigotine, Erythrosine, and Brilliant Green, tend to fade in an alkaline medium.

Table 5. Thin layer chromatography of dyes (free) and their TnOA ion-pairs (IP) on cellulose layers [a]

	Eluant 1		Eluant 2	
Name	IP	Free	IP	Free
Tartrazine	0.41	0.39	0.00	0.00
Quinoline Yellow	0.58	0.60	0.21	0.25
Sunset Yellow	0.78	0.80	0.30	0.31
Azorubine	0.53	0.54	0.11	0.13
Amaranth	0.48	0.51	0.09	0.11
Cochenille Red	0.62	0.66	0.16	0.17
Erythrosine	0.90	0.88	0.55	0.54
Patent Blue V	0.87	0.86	0.48	0.48
Indigotine	0.48	0.50	0.11	0.12
Brilliant Green	0.85	0.84	0.46	0.43
Brilliant Black	0.34	0.33	0.00	0.00

[a] Eluant 1: ethyl acetate–1-propanol–NH_3–water (35 + 35 + 20 + 20); Eluant 2: ethyl acetate–1-butanol–NH_3 (25 + 55 + 25).

Paper Chromatography

Paper chromatography has been applied to the separation of dyes extracted as ion-pairs with quaternary ammonium compounds. Drevon and Laur (3) chromatographed the ion-pairs directly but identified them by comparison with reference dyes also extracted as ion-pairs. Sohár (6) and Vollaire-Salva (7) claimed that it was preferable to back-extract the dyes to an aqueous phase because of the tailing that was observed.

In our experiments, we used 4 mobile phases which are prescribed by the Belgian Food Legislation (13) (Table 8). Some dyes, such as Amaranth, Cochenille Red, and Indigotine, dissociate from TnOA in all 4 systems while most of them have an identical R_f value for the dye and its ion-pair in 2 or 3 systems. For Brilliant Green, this is the case in only one system. Furthermore, the best system, in which all 11 dyes give identical R_f values for the dye and the corresponding ion-pair, is the acidic mobile phase. This indicates that to obtain the dissociation of ion-pairs in a given chromatographic system, polarity of the eluting agent is more important than pH. Very alkaline mobile phases such as the first one cause the rapid discoloration of some dyes (Erythrosine, Indigotine, and Brilliant Green).

Table 6. Thin layer chromatography of dyes (free) and their TnOA ion-pairs (IP) on silica gel layers with mobile phases as in Table 5

	Eluant 1		Eluant 2	
Name	IP	Free	IP	Free
Tartrazine	0.78	0.77	0.00	0.00
Quinoline Yellow	0.83	0.81	0.16	0.17
Sunset Yellow	0.84	0.82	0.15	0.14
Azorubine	0.81	0.84	0.10	0.11
Amaranth	0.73	0.71	0.08	0.10
Cochenille Red	0.75	0.75	0.12	0.12
Erythrosine	0.98	0.99	0.39	0.40
Patent Blue V	0.83	0.81	0.21	0.22
Indigotine	0.91	0.90	0.00	0.00
Brilliant Green	0.80	0.78	0.21	0.22
Brilliant Black	0.82	0.77	0.04	0.06

Table 7. Thin layer chromatography of dyes (free) and their TnOA ion-pairs (IP) on silica gel layers as function of ammonia content of eluant [a]

	Eluant 1		Eluant 2		Eluant 3	
Name	IP	Free	IP	Free	IP	Free
Cochenille Red	0.82	0.80	0.87	0.83	0.94	0.92
Erythrosine	0.98	0.99	0.99	0.98	0.82	0.86
Patent Blue V	0.83	0.80	0.86	0.83	0.82	0.84
Indigotine	0.91	0.88	0.91	0.86	0.99	0.97
Brilliant Green	0.80	0.78	0.82	0.78	0.82	0.84
Brilliant Black	0.82	0.79	0.82	0.79	0.90	0.92

[a] Eluant 1: ethyl acetate–1-propanol–NH_3–water (35 + 35 + 10 + 30); eluant 2: (35 + 35 + 5 + 35); eluant 3: (35 + 35 + 0.1 + 39.9).

Patent Blue V and Brilliant Green show tailing spots in all 4 systems, with no significant difference, however, between the dyes and their ion-pairs. Because some dyes show tailing in these four PC systems, mixtures such as Patent Blue V and Brilliant Green, extracted together, are difficult to separate even when their R_f values are quite different in systems 1, 2, and 4. Tailing is more significant in PC than in TLC, and ion-pairs are more successfully dissociated in TLC; therefore, TLC is preferred for identification of unknown dyes.

High Performance Liquid Chromatography

To avoid demixing caused by the injection of chloroform in a reverse phase ion-pair chromatographic system (1), the chloroform extracts were evaporated to dryness and redissolved in ethanol. The retention times of dyes and extracts are given in Table 9. They demonstrate that in a suitable system both extracted and nonextracted dyes have nearly the same chromatographic behavior. This is demonstrated for Patent Blue V in Figure 1. However, after 20–30 subsequent injections of extracts, peak broadening becomes more and more apparent. After the column is eluted with 100 mL ethanol, the peak shape regains its initial quality. This probably means that tri-*n*-octylamine is adsorbed by the stationary phase and interferes in the retention mechanism.

It is supposed that, upon injection of a TnOA–dye ion-pair, TnOA is replaced in the ion-pair by TBA present in the eluant. Formation of a TBA-dye complex also regulates the retention of free dyes. When TnOA-dye complexes were chromatographed in a mobile phase without TBA, no dyes eluted within 60 min, which also points toward the displacement of TnOA by TBA.

When many extracts must be chromatographed, it is preferable to eliminate the tri-*n*-octylamine to avoid long elution and equilibration times. This may be done either by using a suitable pre-column or by back-extracting the dyes to an aqueous phase. The latter technique was tried since we had previously established the high recoveries obtained with 0.1M solutions of sodium chloride, bromide, iodide, nitrate, and perchlorate (10) and preliminary experiments

Table 8. Paper chromatography on Whatman No. 1 paper of dyes (free) and their TnOA ion-pairs (IP) [a]

	Eluant 1		Eluant 2		Eluant 3		Eluant 4	
Name	IP	Free	IP	Free	IP	Free	IP	Free
Tartrazine	0.37	0.62	0.02	0.01	0.15	0.17	0.07	0.09
Quinoline Yellow	0.03	0.02–0.32	0.14	0.15–0.24	0.26	0.25	0.19	0.21
Sunset Yellow	0.10	0.40	0.33	0.27	0.86	0.30	0.38	0.41
Azorubine	0.10	0.13	0.17	0.16	0.98	0.39	0.41	0.43
Amaranth	0.33	0.32	0.06	0.06	0.13	0.13	0.46	0.47
Cochenille Red	0.47	0.50	0.10	0.09	0.13	0.14	0.17	0.22
Erythrosine	0.05	0.06	0.90	0.50	0.99	0.42	0.53	0.53
Patent Blue V	0.74	0.75	0.77	0.49	0.70	0.65	0.71	0.71
Indigotine	0.18	0.16	0.06	0.09	0.18	0.18	0.06	0.05
Brilliant Green	1.00	0.65	0.96	0.24	0.67	0.41	0.60	0.61
Brilliant Black	1.00	0.60	0.63	0.14	0.16	0.12	0.10	0.11

[a] 1 = 2 g tert. sodium citrate in 100 mL 5% NH_3; 2 = *n*-butanol–ethanol–water–ammonia (50 + 10.5 + 21 + 1); 3 = *n*-butanol–ethanol–water (50 + 26 + 24); 4 = tert. butanol-aq. solution (50 + 50) of 4 g KCl and 240 g propionic acid in 1 L.

Table 9. Retention times of dyes (free) and their TnOA ion-pairs (IP) on MicroPak MCH-10 column with suitable mobile phase

Name	Retention Time, min IP	Free dye
	33% Solvent B	
Tartrazine	3.8	4.0
Quinoline Yellow	6.8 (5.4)(7.9)(12.4)	6.6 (5.2)(7.7)(12.3)
Sunset Yellow	6.3	6.4
Orange GGN	9.8	9.9
Amaranth	4.3	4.2
Cochenille Red	4.4	4.3
Indigotine	3.5 (3.0)	3.4 (3.0)
Brilliant Green	8.8 (10.9)	8.8 (10.8)
Brilliant Black	8.8	9.0
	67% Solvent B	
Erythrosine	23.4	22.9
Patent Blue V	10.1	10.3
Azorubine	4.7	4.6

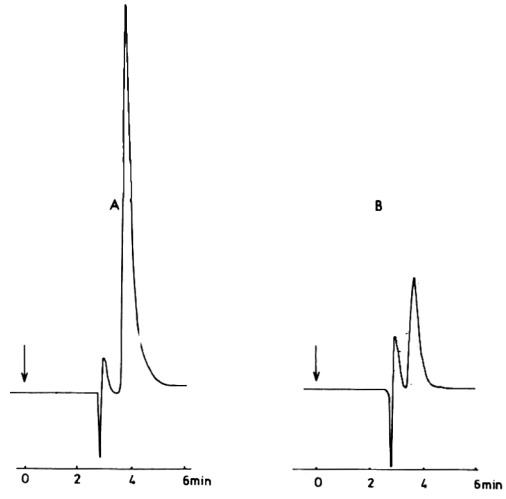


Figure 2. Identification of Tartrazine in pickles. A: TnOA extract of pickles; B: TnOA extract of Tartrazine. Mobile phase: 33% phase B; chart speed 1 cm/min.

indicated no effect of chloride, bromide, iodide, nitrate, and perchlorate ions on the retention time of the dyes. We also studied the resolution between Sunset Yellow and Orange GGN, 2 structural isomers that cannot be separated by TLC and PC. Dye mixtures prepared in pure water and in 0.1M solutions of each salt were chromatographed 6 times in a random sequence to take into account the small fluctuations of the retention times caused by the chromatographic system independent of the injected solutes. Variance analysis showed that resolution between the 2 dyes was not influenced by the solvent used. Identification problems may arise because of the presence of small quantities of subsidiary dyes in Indigotine, Brilliant Green, and Quinoline Yellow. (Retention times of these subsidiary dyes are given between brackets in Table 9.) Dyes cannot be identified solely by measurement of retention times; absorption maxima of the various eluting peaks must also be determined.

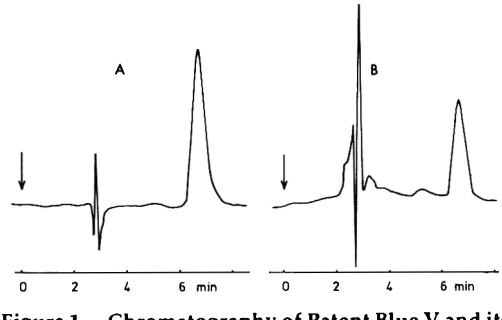


Figure 1. Chromatography of Patent Blue V and its TnOA ion-pair. A: aqueous solution; B: TnOA extract redissolved in ethanol. Mobile phase: 50% phase B; chart speed 1 cm/min.

Application

Tartrazine was extracted from a commercial pickle sample as its ion-pair with TnOA, redissolved in ethanol, and identified by direct injection in the HPLC system with a suitable eluant (Figure 2). Natural oil-soluble colors do not co-extract, so the HPLC system identifies only the synthetic colorant. The identity of the extracted dye was confirmed by TLC on a cellulose layer with ethyl acetate–1-propanol–ammonia–water (35 + 35 + 20 + 20).

Acknowledgment

The authors acknowledge the financial help from the Fund for Scientific Medical Research.

References

(1) Puttemans, M., Dryon, L., & Massart, D. L. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1–8
(2) Schill, G. (1978) *Separation Methods for Drugs and Related Organic Compounds,* Apotekarsocieteten, Stockholm, Sweden
(3) Drevon, B., & Laur, J. (1959) *Ann. Falsif. Fraudes.* **52,** 155–161
(4) Brustier, V., et al. (1966) *Ann. Pharm. Fr.* **24,**

51-56
(5) Trace Materials (Colours) Committee (1963) *Analyst* **88**, 864-871
(6) Sohár, J. (1967) *Z. Lebensm. Unters. Forsch.* **132**, 359-362
(7) Vollaire-Salva, J. (1961) *Ann. Falsif. Exp. Chim.* **54**, 17-20
(8) Perdih, A., & Prihavec, D. (1967) *Z. Lebensm. Unters. Forsch.* **134**, 239-242
(9) Puttemans, M., Dryon, L., & Massart, D. L. (1980) *Anal. Chim. Acta* **113**, 307-314
(10) Puttemans, M., Dryon, L., & Massart, D. L. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 737-744
(11) Schmidt, V. S. (1971) *Amine Extraction*, Israel Program for Scientific Translations Ltd, Jerusalem, Israel
(12) Penner, M. H. (1968) *J. Pharm. Sci.* **57**, 2132-2135
(13) Belgian Food Legislation K.B. 8 (12) 1967

Isolation, Identification, and Determination of Food Dyes Following Ion-Pair Extraction

MARC L. PUTTEMANS, LOUIS DRYON, and DÉSIRÉ L. MASSART
Vrije Universiteit Brussel, Farmaceutisch Instituut, Laarbeeklaan 103, B-1090 Brussels, Belgium

Food dyes are extracted as ion-pairs with tri-*n*-octylamine and tetrabutylammonium. Extraction from aqueous solutions to chloroform is quantitative with 0.1M counter ion concentrations. The dye may be back-extracted to an aqueous phase with chloride, bromide, iodide, nitrate, or perchlorate ions; perchlorate is most successful. The method is applied to the qualitative analysis of dyes in grenadine, pickles, and milk desserts, and quantitative analysis in alcoholic beverages. Commercial samples are analyzed by using a standard addition method.

Various techniques have been used to quantitate dyes: Penner (1) and Lyle and Tehrami (2) measured dyes both by reflectance and by densitometry following thin layer chromatographic (TLC) separation. Drevon and Laur (3) and Sohár (4) extracted dyes as their ion-pairs with quaternary ammonium ions. Drevon and Laur (3) chromatographed the ion-pairs by paper chromatography (PC), eluted the colored spots from the paper sheet with an ammonia solution, and determined the dye content by colorimetry. Sohár (4) back-extracted the dye to an aqueous solution and then measured the dye colorimetrically. This latter technique can be applied only when a single dye is present. Another isolation method, e.g., the use of a liquid anion exchange resin, was chosen by Dolinsky and Stein (5) and Graichen and Molitor (6, 7), who quantitated the dyes by colorimetry.

More recently, dye intermediates were determined in FD&C Red No. 40 (8), FD&C Red No. 2 (9, 10), FD&C Yellow No. 5 (10, 11), and FD&C Yellow No. 6 (12–15) by high performance liquid chromatography (HPLC) using an ion exchange technique. Martin et al. (16) used TLC and reverse phase HPLC to identify dyes isolated from beverages by adsorption on wool yarn. One of the latest developments in HPLC, the ion-pair technique, was applied to dyes by several authors (11, 17–20). One of them (11) described a method for the determination of Tartrazine and its intermediates.

In this paper we describe the application of an ion-pair extraction technique for the quantitative and qualitative determination of dyes in food products. Dyes are extracted by ion-pair formation with one of 2 counter ions: tri-*n*-octylamine (TnOA) or tetrabutylammoniumphosphate (TBA). We have used the former for extraction of dyes (21, 22); the latter was used as a counter ion in the extraction of barbiturates (23), carboxylic acids (24), and penicillins (25). The extracted dyes are then back-extracted to an aqueous phase by a displacement reaction (21). The recovery is measured either by colorimetry or by ion-pair reverse phase HPLC (19).

We describe all aspects of such an analysis for food: pretreatment of the sample, ion-pair extraction, identification, and quantitation. In a previous paper (22) we showed that it is possible to identify dyes by TLC and HPLC by comparing the chromatographic behavior of the TnOA ion-pairs and the free dyes in aqueous solution. In HPLC (22), however, it is preferable to back-extract the dye to an aqueous phase also; therefore, we established the optimum experimental conditions for maximal recovery of the dyes.

Experimental

Apparatus and Reagents

(a) *Spectrophotometer.*—Perkin-Elmer Hitachi 200.

(b) *pH meter.*—Ionalyser 601 and combined glass electrode.

(c) *Liquid chromatograph.*—Varian LC 5020 equipped with Valco loop injector (100 μL loop) and Varian 254 nm UV detector, 300 × 2 mm id column of MCH-10 (MicroPak, 10 μm octadecylsilica); mobile phase 60 mL/h. Peak areas were calculated with a Varian CDS 111 integrator.

(d) *Mobile phases.*—5 mL tetrabutylammonium hydroxide (25% in methanol, Fluka) was diluted to 1 L with mixtures of methanol–phosphate buffer (pH = 7.00 ± 0.05, ionic strength = 0.1): phase A = 30 + 70, phase B = 60 + 40.

Mobile phases were prepared by mixing appropriate amounts of phases A and B. We reported the chromatographic system in a previous paper (19).

(e) *Dyes.*—Purchased from P. Entrop (Machelen, Belgium) and used as received.

Received July 15, 1981. Accepted November 20, 1981.

0004-5756/82/6503-0737-08$01.00

(f) *Counter ions.*—Tri-*n*-octylamine (Aldrich) and tetrabutylammonium phosphate (0.5M solution in phosphate buffer, pH 7.5, Altex) were used as received.

(g) *Buffers.*—With constant ionic strength (0.1), prepared as follows: pH = 5.5: 1.26 g $Na_2HPO_4.2H_2O$ and 24.65 g $NaH_2PO_4.H_2O$ dissolved in double-distilled water and diluted to 2 L; pH = 7.0: 9.38 g $Na_2HPO_4.2H_2O$ and 5.77 g $NaH_2PO_4.H_2O$ dissolved in double-distilled water and diluted to 2 L. If necessary, pH was adjusted to 5.50 ± 0.05 or 7.00 ± 0.05 with 0.1M sodium hydroxide. Buffers used for batch extractions, and 0.1M sodium salt solutions used for back-extractions of dyes, were saturated with chloroform which was ethanol-free and saturated by repeated shaking with water.

(h) *TLC system.*—Cellulose precoated plates, 20 × 20 cm (Merck), 0.1 mm thick. Eluant: ethyl acetate–*n*-propanol–ammonia–water (35 + 35 20 + 20).

Extraction

Equal volumes (10 mL) of aqueous and organic phases were shaken 30 min in centrifuge tubes as follows: For extractions with TnOA, 10 mL dye solution (10^{-5}M or 5.10^{-5}M), buffered at pH 5.5 with phosphate buffer (ionic strength = 0.1), was shaken with 10 mL 1.0M (or 0.10M) TnOA solution in chloroform. For extractions with TBA, 5 mL dye solution (2.10^{-5}M or 10^{-4}M), buffered at pH 7.0 with phosphate buffer (ionic strength = 0.1), was mixed with 5 mL 0.2M (or 0.02M) TBA solution and 10 mL chloroform. Back-extractions were also performed with equal phase volumes (7 mL each). Phases were separated after centrifugation, and the extraction yield was determined by measuring the dye concentration in the aqueous phase. All extractions were carried out in triplicate.

Analysis of Commercial Samples

(a) *Grenadine and alcoholic beverages.*—(*1*) *Preliminary experiment.*—A 2 mL sample was diluted with 10 mL phosphate buffer (pH 5.5; ionic strength 0.1). Dyes were extracted with 5 mL 0.1M TnOA in chloroform and identified by direct TLC of the TnOA extract (22). Dyes were then back-extracted to 0.1M sodium perchlorate (or other salt) solution by shaking 3 mL chloroform layer with an equal volume of the salt solution. Identity of the extracted dyes was confirmed by HPLC of the perchlorate phase, and dye content was estimated by use of a calibration curve.

(*2*) *Quantitative determination by standard addition.*—To 2 mL samples, 50, 100, and 150% of the estimated amounts of dyes were added; a suitable internal standard was also added. If the sample contained one or more of the dyes Tartrazine, Quinoline Yellow, Cochenille Red, Indigotine, or Brilliant Black, the procedure as listed under (a) (*1*) was followed. If one or more of the dyes Sunset Yellow, Azorubine, Amaranth, Erythrosine, Patent Blue or Brilliant Green was present, an alternative method was used, as follows: Ten mL 0.05M TBA solution in phosphate buffer (pH 7.0) was added to 2 mL sample, which was then extracted with 5 mL chloroform. Back-extraction proceeded as in (a) (*1*). Determinations were carried out in triplicate.

(b) *Cochineal in alcoholic beverages.*—A cochineal-containing sample, after standard addition, was then extracted with TnOA and back-extracted with perchlorate as mentioned above. A 2 mL portion of the perchlorate extract was

Table 1. Percent of extraction of dyes into chloroform from aqueous buffer solutions with different concentrations of TnOA or TBA

Name	CI No.	Concn (M)	TnOA (pH = 5.5) 1.0M	TnOA (pH = 5.5) 0.10M	TBA (pH = 7.0) 1.0M	TBA (pH = 7.0) 0.10M
Tartrazine	19140	5×10^{-5}	100.0 ± 0.2	100.0 ± 0.3	96.4 ± 0.8	22.0 ± 0.3
Quinoline Yellow	47005	5×10^{-5}	99.8 ± 0.7	99.7 ± 0.5	98.9 ± 0.7	36.2 ± 0.4
Sunset Yellow	15985	5×10^{-5}	99.8 ± 0.6	99.5 ± 0.8	97.3 ± 0.5	43.4 ± 0.4
Azorubine	14720	5×10^{-5}	99.8 ± 0.7	98.9 ± 0.9	100.0 ± 0.6	56.2 ± 0.5
Amaranth	16185	5×10^{-5}	99.6 ± 0.4	99.6 ± 0.5	98.5 ± 0.5	40.8 ± 0.4
Cochenille Red	16255	5×10^{-5}	100.0 ± 0.9	99.1 ± 0.7	97.3 ± 0.6	39.5 ± 0.4
Erythrosine	45430	1×10^{-5}	98.1 ± 0.5	96.4 ± 0.6	99.1 ± 0.7	98.6 ± 0.5
Patent Blue V	42051	5×10^{-5}	99.3 ± 0.6	99.4 ± 0.8	99.3 ± 0.8	42.3 ± 0.4
Indigotine	73015	1×10^{-5}	96.2 ± 0.4	79.0 ± 0.8	95.0 ± 0.7	31.5 ± 0.4
Brilliant Green	44090	1×10^{-5}	100.0 ± 0.3	99.5 ± 0.4	97.8 ± 0.5	42.5 ± 0.4
Brilliant Black	28440	1×10^{-5}	100.0 ± 0.4	99.5 ± 0.7	96.3 ± 0.6	37.8 ± 0.3

treated with 250 μL 5% uranyl acetate solution in double-distilled water and diluted to 25 mL with 0.1M sodium perchlorate. The absorbance at 621 nm was measured in 50 mm cells. Determinations were carried out in triplicate.

(c) *Pickles and canned cherries.*—Five g pickles or dried cherries was macerated 1 h at 50°C in 100 mL 0.1% ammonia solution. The solution was filtered and the pH was adjusted to 5.5 with 0.1% phosphoric acid. The dyes were extracted with 10 mL 0.1M TnOA in chloroform.

(d) *Milk desserts (rice milk).*—Five g dried milk dessert was transferred to a 35 cm × 2 cm id glass chromatography tube, and dyes were eluted with 100 mL methanol–ammonia (95 + 5). The eluate was evaporated to dryness, the residue was dissolved in 20 mL phosphate buffer (pH = 5.5, ionic strength: 0.1), and the dyes were extracted with 5 mL 0.1M TnOA solution in chloroform and then back-extracted with 3 mL 0.1M sodium perchlorate.

Results and Discussion

Selection of Counter Ion

As shown in Table 1, all dyes investigated were extracted with 0.1M and 0.01M solutions of both counter ions. As we demonstrated (22), the TnOA extraction is quite dependent on the pH of the solution. Indeed, the acid dye must be ionized while the amine must be protonated. The optimum pH value lies between 5 and 6. On the other hand, TBA is ionized over the whole pH range and the choice of pH is less critical.

Extractions with 0.1M counter ion concentrations give satisfactory results for both counter ions. At a 0.01M concentration, the difference between TnOA and TBA becomes apparent. While TnOA still gives quantitative extraction, except for Indigotine, TBA gives much lower extraction yields. This difference can be easily explained by the higher degree of hydrophobicity of TnOA which has 24 carbon atoms vs 16 for TBA. Consequently, one may state that TnOA is a better counter ion than TBA in the extraction of food dyes, except Erythrosine is extracted somewhat better with TBA.

Selection of Displacing Ion

Back-extractions were tried first with TnOA dye extracts. Dyes were extracted with a 0.1M TnOA solution in chloroform and this solution was then shaken with an equal volume of a 0.1M salt solution. Various competing ions were tested as their sodium salts: chloride, bromide,

Table 2. Percent of back-extraction of dyes extracted as ion-pairs with TnOA or TBA from aqueous 0.1M salt solutions, or 0.1M sodium perchlorate

Name	Concn (M)	TnOA					TBA		Sodium perchlorate	
		Cl^-	Br^-	I^-	NO_3^-	ClO_4^-	NO_3^-	ClO_4^-	TnOA	TBA
Tartrazine	5×10^{-5}	100.0 ± 0.4	78.3 ± 0.4	72.1 ± 0.4	80.0 ± 0.5	98.3 ± 0.4	52.8 ± 0.4	99.3 ± 0.5	98.3	96.0
Quinoline Yellow	5×10^{-5}	68.3 ± 0.5	94.1 ± 0.8	99.6 ± 0.7	93.5 ± 0.7	99.1 ± 0.5	90.3 ± 0.6	99.0 ± 0.7	98.9	97.9
Sunset Yellow	5×10^{-5}	69.4 ± 0.4	96.7 ± 0.5	99.4 ± 0.8	97.4 ± 0.8	99.3 ± 0.6	95.2 ± 0.7	98.7 ± 0.6	99.1	96.0
Azorubine	5×10^{-5}	4.0 ± 0.1	67.5 ± 0.3	88.4 ± 0.6	6.6 ± 0.1	95.2 ± 0.5	15.8 ± 0.3	95.3 ± 0.7	95.0	95.3
Amaranth	5×10^{-5}	23.3 ± 0.2	94.3 ± 0.6	96.2 ± 0.7	93.0 ± 0.8	100.0 ± 0.8	98.2 ± 0.7	100.0 ± 0.7	99.6	98.5
Cochenille Red	5×10^{-5}	86.6 ± 0.5	100.6 ± 0.7	101.0 ± 0.8	100.1 ± 0.6	99.7 ± 0.7	100.0 ± 0.5	99.8 ± 0.6	100.0	97.3
Erythrosine	1×10^{-5}	5.5 ± 0.1	0.3 ± 0.0	1.1 ± 0.0	0.0 ± 0.0	6.3 ± 0.1	5.5 ± 0.1	29.0 ± 0.2	6.2	28.7
Patent Blue V	5×10^{-5}	5.6 ± 0.1	78.2 ± 0.4	79.9 ± 0.6	86.8 ± 0.6	92.1 ± 0.7	86.3 ± 0.7	94.3 ± 0.8	91.5	93.6
Indigotine	1×10^{-5}	89.8 ± 0.5	96.3 ± 0.7	96.4 ± 0.7	97.3 ± 0.6	98.0 ± 0.6	15.0 ± 0.2	15.0 ± 0.1	94.2	14.3
Brilliant Green	1×10^{-5}	56.3 ± 0.3	49.5 ± 0.4	46.9 ± 0.4	48.9 ± 0.5	64.3 ± 0.6	54.0 ± 0.3	82.5 ± 0.7	64.4	80.7
Brilliant Black	1×10^{-5}	98.0 ± 0.7	90.7 ± 0.6	96.7 ± 0.8	89.9 ± 0.8	93.6 ± 0.7	88.3 ± 0.7	96.3 ± 0.7	93.6	92.7

Table 3. Recovery of 50 ppm dyes added to wine aperitif, extracted with TnOA or TBA, and back-extracted with 0.1 M sodium perchlorate

	TnOA		TBA	
Name	Rec., %	Source of loss	Rec., %	Source of loss
Tartrazine	98.1 ± 2.9	—	53.1 ± 2.0	extn
Quinoline Yellow	100.2 ± 5.1	—	96.7 ± 1.0	—
Sunset Yellow	63.2 ± 4.0	back-extn	92.9 ± 3.1	extn
Azorubine	38.7 ± 3.9	back-extn	90.0 ± 4.0	extn
Amaranth	63.3 ± 1.5	back-extn	98.3 ± 4.6	—
Cochenille Red	87.3 ± 4.3	back-extn	88.2 ± 4.2	extn
Erythrosine	5.6 ± 0.3	extn + back-extn	18.6 ± 0.8	extn + back-extn
Patent Blue V	12.3 ± 2.6	extn + back-extn	66.1 ± 2.3	extn
Indigotine	100.1 ± 2.7	—	39.9 ± 2.3	back-extn
Brilliant Green	24.5 ± 3.1	extn + back-extn	67.0 ± 7.2	extn
Brilliant Black	65.1 ± 2.8	back-extn	33.1 ± 1.2	extn

iodide, nitrate, perchlorate, phosphate, sulfate, borate, and citrate. The latter 4 were rejected immediately because of low (10–50%) extraction yields for the tested dye (Quinoline Yellow). As listed in Table 2, chloride, bromide, iodide, nitrate, and perchlorate give sufficient back-extraction yields. The latter 3 give nearly quantitative back-extractions except for Erythrosine and Azorubine.

Gérin and Fresco (26) found that the partition constants of tri-*n*-octylamine chloride, bromide, iodide, and perchlorate between water and nitrobenzene increase in the order $Cl^- < Br^- < I^- < ClO_4^-$; therefore, we studied whether the displacement of dyes by these ions follows the same order. Data listed in Table 2 indicate that this is the case for 7 of the 11 dyes investigated (Quinoline Yellow, Sunset Yellow, Azorubine, Amaranth, Cochenille Red, Patent Blue, and Indigotine).

Extractions with sodium iodide are less useful in dye analysis, partly because iodide is easily oxidized to iodine but mostly because it gives an interfering solvent peak in the ion-pair reverse phase HPLC system (18, 22). From the back-extraction experiments, it may be concluded that nitrate and perchlorate are best suited for displacement of dyes from their TnOA complexes and their subsequent determination by HPLC. These salts were likewise investigated for back-extraction of dyes from TBA complexes. Results (Table 2) indicate that recoveries are similar or higher than with TnOA, especially for Azorubine and Erythrosine and confirm that TBA is a less powerful counter ion for the extraction of dyes and perchlorate is preferred as displacing ion. Considering overall recoveries (Table 2), for the dyes Azorubine, Erythrosine, Patent Blue, and Brilliant Green, it is preferable to extract with TBA instead of TnOA.

Recoveries from Alcoholic Beverages

Dyes were added to dye-free alcoholic beverages at 50 ppm. Recoveries of dyes added to

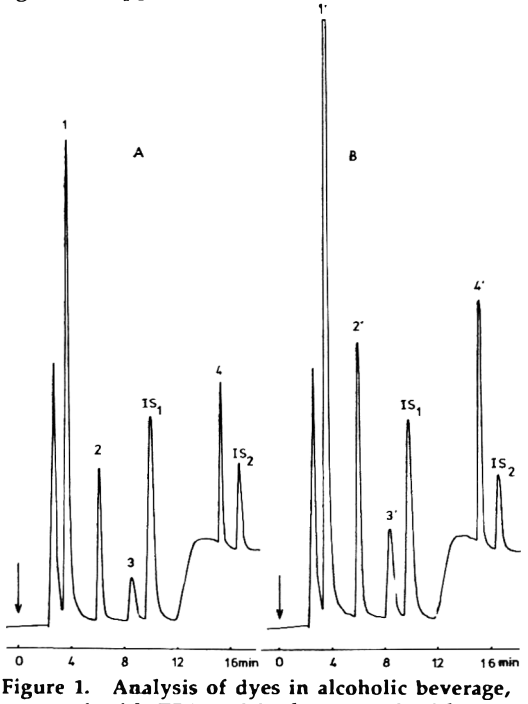


Figure 1. Analysis of dyes in alcoholic beverage, extracted with TBA and back-extracted with perchlorate:

A, Chromatogram of sample (dyes 1, 2, 3, and 4) + internal standards (IS_1-Orange GR, IS_2-Patent Blue V); B, chromatogram of sample + internal standards + 200 μL stock solution: 1′ = 1 = Tartrazine, 2′ = 2 = Sunset Yellow, 3′ = 3 = Brilliant Black, 4′ = 4 = Azorubine. Mobile phase gradient scheme: 0, 7, 7.1, and 14 min = 15, 15, 100, and 100% B.

Table 4. Analysis results of commercial alcoholic beverages

Sample	Name	Amount, ppm	Method
1	Tartrazine	28.9 ± 0.4	HPLC
	Sunset Yellow	9.5 ± 0.2	HPLC
	Azorubine	13.9 ± 0.3	HPLC
	Brilliant Black	3.4 ± 0.3	HPLC
2	Patent Blue V	51.7 ± 1.6	colorimetry
3	Sunset Yellow	172.3 ± 1.5	colorimetry
		170.5 ± 3.6	HPLC
4	Tartrazine	18.8 ± 1.0	HPLC
	Patent Blue V	11.2 ± 1.3	HPLC
5	Tartrazine	17.5 ± 1.2	HPLC
	Patent Blue V	0.60 ± 0.05	HPLC
6	Amaranth	11.9 ± 0.3	HPLC
	Cochineal	130.6 ± 11.3	colorimetry

beverages such as elixirs consisting mainly of water, alcohol, sweeteners, flavor components, and colorants were similar to those obtained from aqueous solutions. However, recoveries of dyes added to more complex matrices such as vermouths and aperitifs manufactured from wine were lower (Table 3). With TnOA as counter ion, losses are mostly due to incomplete back-extraction; with TBA as counter ion, low yields are mostly due to incomplete primary extraction. For Erythrosine, Patent Blue, and Brilliant Green, the primary extraction yields are low although almost no color remains in the aqueous phase. The loss is due to adsorption of the dyes to wine components precipitated at the interphase between the chloroform and aqueous phases. Since adsorption of dyes was less significant in alkaline medium, we centrifuged the sample after addition of ammonia. The pH of the supernatant liquid was adjusted and extraction was carried out as usual. Extraction yield improved substantially for Erythrosine, Patent Blue, and Brilliant Green; almost 90% of the added dye was now extracted both with TnOA and TBA. Although it is difficult to choose a preferred counter ion for overall quantitative extraction for all dyes, TBA is preferred for Sunset Yellow and Amaranth.

Analysis of Commercial Alcoholic Beverages

TnOA-extracted dyes are first identified by direct TLC of the TnOA ion-pairs. Dyes are then back-extracted with perchlorate and their identity is confirmed by HPLC. The dye content of the perchlorate extract is determined tentatively by means of a calibration curve. With this information, a new extraction is carried out, if necessary, with TBA instead of TnOA.

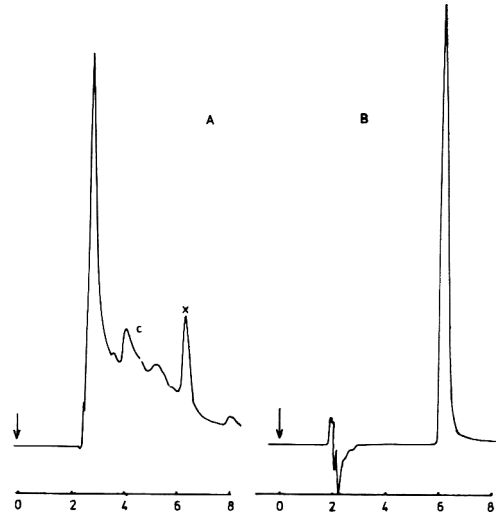


Figure 2. HPLC analysis of Sample 6, extracted with TnOA and back-extracted with perchlorate: A, sample, c = Cochineal, x = unknown dye; B, Amaranth. Mobile phase: 5% B.

The sample is also analyzed using a standard addition method: 50, 100, and 150% of the amount of dye found in the sample by preliminary experiment is added to sample aliquots. If only one dye is present, the dye content in the extract is determined by colorimetry. In other cases, dyes are quantitated by HPLC at a suitable mobile phase composition. As an illustration the analysis of one sample is given below: Upon extraction of the dyes with 0.1M TnOA in chloroform and back-extraction with 0.1M sodium

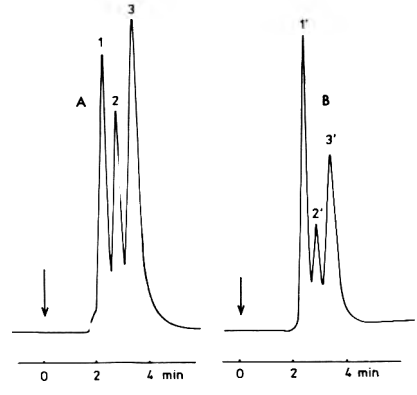


Figure 3. Identification of dyes in a grenadine sample: A, sample with unknown dyes 1, 2, and 3; B, mixture of reference dyes: 1 = 1′ = Amaranth, 2 = 2′ = Cochineal Red, 3 = 3′ = Quinoline Yellow. Mobile phase: 25% phase B; chart speed 1 cm/min.

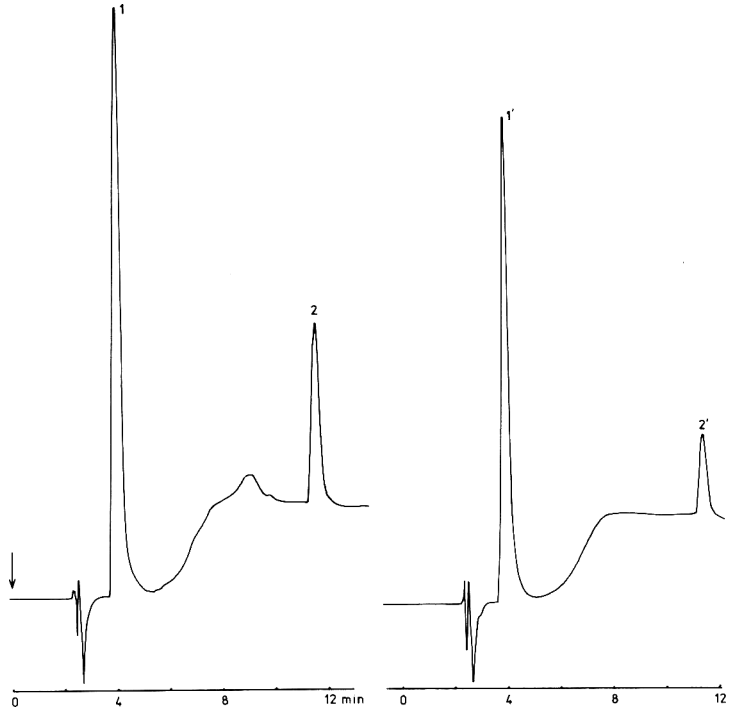


Figure 4. Identification of dyes in a brandy sample (Sample 4): Left, sample with unknown dyes 1 and 2; right, mixture of reference dyes: 1 = 1' = Tartrazine, 2 = 2' = Patent Blue V. Mobile phase gradient scheme: 0, 2, 10, and 10.1 min = 15, 100, 100, and 15% B; chart speed 1 cm/min.

perchlorate, the sample was shown to contain 30 ppm Tartrazine, 17.5 ppm Sunset Yellow, 1.25 ppm Azorubine, and 8.5 ppm Brilliant Black. A dye stock solution was prepared containing 30 mg Tartrazine, 17.5 mg Sunset Yellow, 5 mg Azorubine, and 8.5 mg Brilliant Black in 100 mL double-distilled water. To 2 mL sample, 0 μL, 100 μL, 200 μL, or 300 μL of the dye stock solution together with 200 μL of an internal standard solution (10 mg Orange GR and Patent Blue in 100 mL double-distilled water) were added. Because the chloroform phase was still red after back-extraction with perchlorate, due to incomplete extraction of Azorubine, TnOA was replaced by TBA to increase over-all extraction yield. The extraction procedure was repeated in the new system, i.e., with standard addition and internal standard so that several kinds of errors such as nonquantitative extraction yields, volume variations due to the presence of alcohol in a water–chloroform system, and incorrect injection volumes, were avoided.

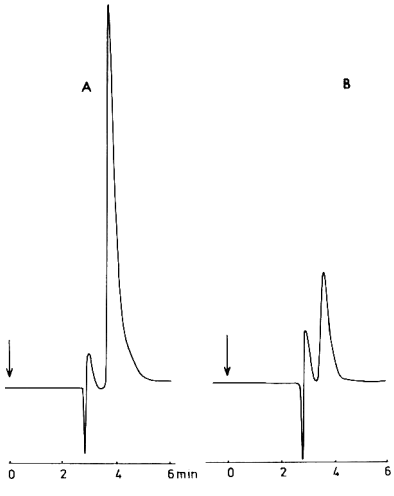


Figure 5. Identification of Tartrazine in pickles: A, TnOA extract of pickles; B, TnOA extract of Tartrazine. Mobile phase: 33% B; chart speed 1 cm/min.

Extracts were chromatographed in a gradient HPLC system (Figure 1), and areas of the corresponding peaks were calculated by integrator. Peak areas were divided by the peak area of the internal standard. Tartrazine, Sunset Yellow, and Brilliant Black were calculated with Orange GR as internal standard; the concentration of Azorubine was obtained relative to Patent Blue.

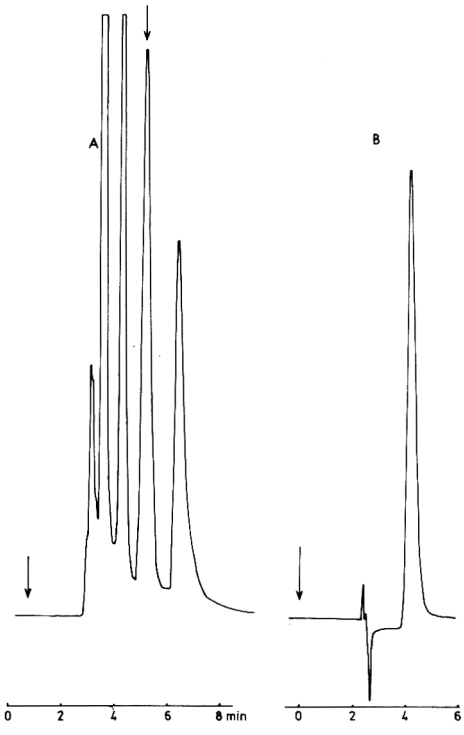


Figure 6. Identification of Tartrazine in milk desserts (rice milk): A, perchlorate extract of rice milk; B, aqueous solution of Tartrazine.

Relative peak areas were plotted vs the added amount of dye. In practice, the value of the intercept was calculated through a curve-fitting program.

Table 4 shows the results obtained for this sample and a few others. The large amount of Sunset Yellow found by colorimetry for Sample 3 was confirmed by HPLC. Because Sample 6 contains Amaranth and Cochineal, a natural acidic dye, it was necessary to look for an adequate quantitation method for Cochineal in the presence of Amaranth. Amaranth can be determined by HPLC but Cochineal gives a tailing peak by the HPLC system used (Figure 2). Direct colorimetry of the extract is also excluded because of the presence of the other red dye. Therefore, we investigated the applicability of a qualitative test, the reaction of Cochineal with uranyl acetate (27), to quantitative determinations. It appeared that the intensity of the green reaction between Cochineal and uranyl acetate increases linearly with an increase of the amount of Cochineal and is independent of the presence of Amaranth (5, 10, and 50 ppm Amaranth were added). The determination of cochineal in a commercial sample was carried out by the standard addition technique, since the overall extraction yield was only 75%.

Qualitative Analysis of Various Food Products

For identification of dyes in liquid samples, the procedure is the same as for alcoholic beverages. Chromatograms for a grenadine and an alcoholic beverage are presented in Figures 3 and 4. For solid foods such as pickles and canned cherries, dyes must be solubilized first by maceration, then extracted and identified by TLC and HPLC. Identification of Tartrazine in pickles is shown in Figure 5 (22). For rice milk, a food high in carbohydrate, fat, and protein, it is impossible to apply the maceration technique. Column chromatography is used to separate the dye from the matrix, followed by TnOA extraction. The extracted dye is identified in the perchlorate phase by TLC and HPLC (Figure 6).

Acknowledgments

The authors acknowledge financial help of the Fund for Scientific Medical Research, and thank A. Langlet-De Schryver and K. Broothaers-Decq for technical assistance.

REFERENCES

(1) Penner, M. H. (1968) *J. Pharm. Sci.* **57,** 2132-2135
(2) Lyle, S. J., & Tehrami, M. S. (1979) *J. Chromatogr.* **175,** 163-168
(3) Drevon, B., & Laur, J. (1959) *Ann. Falsif. Fraud.* **52,** 155-161
(4) Sohár, J. (1967) *Z. Lebensm, Unters. Forsch.* **132,** 359-362
(5) Dolinsky, M., & Stein, C. (1962) *J. Assoc. Off. Agric. Chem.* **45,** 767-769
(6) Graichen, C., & Molitor, J. C. (1963) *J. Assoc. Off. Agric. Chem.* **46,** 1022-1029
(7) Graichen, C. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 278-282
(8) Singh, M. (1974) *J. Assoc. Off. Anal. Chem.* **57,** 219-220
(9) Singh, M. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 173-175
(10) Bailey, C. J., Cox, E. A., & Springer J. A. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1404-1414
(11) Wittmer, D. P., Nuessle, N. O., & Haney W. G. (1975) *Anal. Chem.* **47,** 1422-1423
(12) Marmion, D. M. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 168-172
(13) Singh, M. (1974) *J. Assoc. Off. Anal. Chem.* **57,** 358-359
(14) Bailey, J. E., & Cox, E. A. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 609-613
(15) Marmion, D. M. (1975) *J. Assoc. Off. Anal. Chem.* 58, 719-724
(16) Martin, G E., Tenenbaum, M., Alfonso, F., & Dyer, R. H. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 908-910

(17) Knox, J. H., & Laird, G. R. (1976) *J. Chromatogr.* **122,** 17–34
(18) Masiala-Tsobo, C., Lagrange, G., & Dorlet C. (1979) *Anal. Lett.* **12,** 477–490
(19) Puttemans, M. L., Dryon, L., & Massart D. L. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1–8.
(20) Chudy, J., Crosby, N. T., & Patel, I. (1978) *J. Chromatogr.* **154,** 306
(21) Puttemans, M. L., Dryon, L., & Massart, D. L. (1980) *Anal. Chim. Acta* **113,** 307–314
(22) Puttemans, M. L., Dryon, L., & Massart, D. L. (1982) **65,** 730–736
(23) Borg, K. O., & Schill, G. (1968) *Acta Pharm. Suecica* **5,** 323–334
(24) Modin, R., & Tilly, A. (1968) *Acta Pharm. Suecica* **5,** 311–322
(25) Modin, R., & Schröder-Nielsen, M. (1971) *Acta Pharm. Suecica* **8,** 573–584
(26) Gérin, M., & Fresco, J. (1978) *Anal. Chim. Acta* **97,** 165–176
(27) Hart, F. L., & Fisher H. J. (1971) *Modern Food Analysis,* Springer Verlag, New York, NY

THICKENING AGENTS

Electrophoretic Method for Qualitative and Quantitative Analysis of Gelling and Thickening Agents

UDO PECHANEK, GERNOT BLAICHER, WERNER PFANNHAUSER, and HERBERT WOIDICH
Forschungsinstitut der Ernährungswirtschaft, Blaasstrasse 29, 1190 Wien, Austria

An electrophoretic method has been developed for qualitative and quantitative determination of all common thickening and gelling agents. Thickeners were identified by their migration behavior, their staining ability, and the characteristic shapes of their electrophoretic zones, and then quantitated in a scanner. A method is also reported for isolating thickeners from food, including removal of fat and dyes by dioxane, enzymatic starch degradation, removal of protein by trichloroacetic acid, and precipitation of the polysaccharides by absolute ethanol. Isolation of gelatin is also described.

With the increased production of foods like instant puddings, creams, and drinks, and precooked frozen meals, the use of gelling and thickening agents has also increased. The natural products originating from algae or plant seeds, exudates, and extracts show multifunctional qualities as stabilizers, thickeners, and binding and suspending agents.

Until now, few authors have reported on the analytical aspect of these substances. The usual analytical methods apply microscopic examinations and unspecific precipitation reactions (1–4). Other attempts to analyze thickeners include hydrolysis of the polysaccharides before determination of their components, and direct analysis (without hydrolysis or derivatization). Constituents are then identified by methods like thin layer chromatography (5, 6), paper chromatography (7), gas chromatography (8–12), thin layer electrophoresis (13), or polarography (13). These analyses are tedious and lengthy, and give acceptable results only if a single thickener is present, because of similarities between thickener components and the problem of hydrolysis.

Some good results were obtained in experiments using the following methods: separation on DEAE-cellulose (14) and gas chromatographic analysis of the constituents, electrophoresis with cellulose acetate membranes (15), moving boundary electrophoresis (16), electrophoresis with polyacrylamide gels (17, 18), serological identification (17), or gel permeation high pressure liquid chromatography (HPLC) (19). The best results were obtained by Padmoyo and Miserez (15) who separated some thickeners by using electrophoresis with cellulose acetate membranes before identification by different dyes.

We tried to develop a method for qualitative and quantitative determination of all common thickeners without hydrolysis or derivatization. We used diluted polyacrylamide gels as well as agarose layers and, finally, cellulose acetate membranes. We identified thickeners based on these 3 criteria: migration on the layer in the electric field, ability to be stained, and shape of electrophoretic zone.

METHOD

Apparatus

(a) *Thin layer electrophoresis equipment.*—Beckman Microzone cell, type R-101, with Beckman Microzone sample applicator.

(b) *Power supply.*—Beckman type R-120.

(c) *Scanner*—Zeiss TL Photometer PMQII, nonmonochloromatographic part M4QIII, XY-recorder HP.

(d) *Ultrasonic bath.*

Reagents

(a) *Cellulose acetate membranes.*—145 × 57 mm (Beckman).

(b) *Standard thickener substances.*—Sigma. Prepare solutions of 0.5–0.001% (as given Table 1) in water by stirring on 100°C heating plate. Filter before use. For agar-agar, dissolve in 0.1N HCl, heat, and filter. Neutralize with 0.1N NaOH.

(c) *Stains.*—Methylene Blue and Amido Black 10B (Merck); Toluidine Blue O and Fuchsin (Serva); commercially prepared Fuchsin reagent (Merck). Prepare 0.2% Methylene Blue; 0.2%

0004-5756/82/6503-0745-08$01.00

Table 1. Optimum concentrations and application frequency for thickeners and particular stains

Thickener	Concn, %	Application frequency	Amt, ng
		Fuchsin	
Alginic acid	0.1	5–8	1250–2000
Ca-Alginate	0.1	3–6	750–1250
Carob (locust bean gum)	0.02	4–6	200–300
Guar	0.01	5–7	125–175
Gum arabic	0.025	2–4	127–254
Gum ghatti	0.5	1–3	1250–3750
Gum karaya	0.5	2–5	2500–6250
K-Alginate	0.1	5–7	1250–1750
Na-Alginate	0.1	5–7	1250–1750
Pectin	0.25	1–3	625–1875
Propylene glycol alginate	0.25	5–8	3125–5000
Starch, soluble	0.1	3–5	750–1250
Starch, modified	0.1	8–12	2000–3000
Tragacanth	0.125	3–5	937.5–1562.5
		Toluidine Blue	
Agar–agar	0.5	6–16	10 000–20 000
Alginic acid	0.05	2–4	250–500
Ca-Alginate	0.05	1–3	125–375
Carrageenans	0.05	2–6	250–750
Carboxy methyl cellulose	0.05	1–4	125–500
Gum arabic	0.125	2–6	625–1865
Gum ghatti	0.5	2–5	2500–6250
Gum karaya	0.5	1–5	1250–6250
K-Alginate	0.05	2–6	250–750
Na-Alginate	0.05	1–4	125–500
Pectin	0.125	1–5	312.5–1562.5
Propylene glycol alginate	0.25	2–4	1250–2500
Tragacanth	0.125	4–6	1250–1875
		Methylene Blue	
Agar–agar	0.5	6–10	7500–12 500
Alginic acid	0.1	2–4	500–1000
Ca-Alginate	0.1	3–5	750–1250
Carrageenans	0.05	3–5	375–625
Carboxy methyl cellulose	0.05	6–8	750–1000
Gum arabic	0.25	3–5	1875–3125
Gum ghatti	0.5	6–8	7500–10 000
Gum karaya	0.5	6–8	7500–10 000
K-Alginate	0.05	2–4	250–500
Na-Alginate	0.1	1–3	250–750
Pectin	0.25	2–4	1250–2500
Propylene glycol alginate	0.25	3–5	1875–3125
Tragacanth	0.25	8–10	5000–6250
		Amido Black 10B	
Gelatin	0.1	2–5	500–1250

Toluidine Blue O; and 1% Amido Black 10B (or 0.1% in methanol–acetic acid (9 + 1)) in water and filter before use. To prepare Fuchsin reagent (Schiff's reagent), dissolve 1 g in ca 80 mL boiling water, and stir vigorously 90 min. Cool under running tap water. Add 1 mL concentrated HCl and 2 g $K_2S_2O_5$. Dilute with water to 100 mL and continue to stir 1 h. Let stand overnight. About 25 min before use, incubate 30 mL reagent with 1.2 g powdered charcoal, filter, and use clear, colorless solution for staining.

(d) *Periodic acid.*—Dissolve 2 g periodic acid in 10 mL water. Add 90 mL denatured alcohol. Prepare freshly daily. Before using Fuchsin reagent, dip sample membrane into periodic acid 5 min.

(e) *Trichloroacetic acid solution.*—50% in water.

(f) *Boric acid buffer* (15).—Dissolve 12.4 g boric acid and 4 g NaOH in 1 L water (pH = 10, ionic strength = 0.13).

(g) *Sodium carbonate buffer* (15).—Dissolve 3.9 g Na_2CO_3 and 6.3 g $NaHCO_3$ in 1 L water (ionic strength = 0.15).

(h) *Enzymes.*—α-Amylase (Merck); amyloglucosidase (Merck).

Isolation of Polysaccharides

To remove all food components except thickeners, the process consists of 4 steps: removing fat, starch, and proteins, and precipitating thickeners. Depending on the concentration of components in the sample, the isolation procedure can be modified by repeating a step (fat and color extraction), increasing incubation time or increasing enzyme concentration (starch removal), or increasing the amount of chemicals (protein precipitation). A step may be omitted when a food does not contain a component. As given in the procedure, use a thermostated water bath and laboratory centrifuge (max 3000 rpm), equipped with 80 mL centrifuge tubes. Cover tubes with watch glasses during all heating steps.

Preparation of Samples

Dissolve 0.5–10 g solid sample in appropriate amount of water by heating in 100°C water bath.

Extraction of Fat and Dyes

Add 40 mL dioxane and hold 5 min at 60°C. Centrifuge at 3000 rpm. Decant and repeat dioxane treatment for intensely colored samples or those containing more than 10% fat. Wash residue twice with 10 mL portions of 70% ethanol.

If washings are still colored, repeat washings until liquid is colorless. Dry residue carefully under stream of nitrogen. Dissolve in 5–10 mL water by warming to 60–70°C and mixing in ultrasonic bath.

Starch Degradation

Add freshly prepared solution of α-amylase (0.5–1 mg in 1 mL water) and let stand at room temperature 30–60 min. Warm to 50°C and add solution of freshly prepared amyloglucosidase (0.2–1 mg in 1 mL water) and hold for 1–2 h.

Precipitation of Protein

Warm to 60°C and add urea (1–5 g), until solution becomes more or less transparent. Complete dissolution at this temperature. Add drops of 50% trichloroacetic acid (1–5 mL) until precipitation is complete. Hold at this temperature 15 min; then centrifuge. Quantitatively pour liquid phase into another 80 mL centrifuge tube.

Precipitation of Polysaccharides

Add 50 mL absolute ethanol and let stand overnight. Decant, and wash residue with 5–10 mL portions of 70% ethanol; then dry under stream of nitrogen. Add small amounts of water (1–2 mL) with stirring and dissolve residue at 100°C. Collect these portions in 5 or 10 mL volumetric flask. Complete dissolution in ultrasonic bath.

Electrophoretic Procedures

(a) *Polyacrylamide gel electrophoresis.*—Conditions as follows: 5% polyacrylamide + 0.83% *N,N'*-methylenebisacrylamide, diluted 2 + 1; boric acid buffer; current 40 mA; run time 2 h; cell as described by Ebermann and Barna (23).

(b) *Agarose gel electrophoresis.*—Conditions as follows: agarose gels (Beckman); boric acid buffer; 100 V; run time 20 min; Beckman Microzone cell R-101.

(c) *Cellulose acetate membrane electrophoresis.*—Wet membrane only along sides of 2 rows of holes, using boric acid buffer, and fix it on cell bridge. Put membrane onto self-made wooden block (4 cm high, 2 cm wide, same length as cell bridge, with 1 cm border on left and right side to keep cell bridge in position) outside cell vessel and adjust cell cover above. Wet the 8 possible spots on the sheet with 0.25 μL boric acid buffer, using Microzone applicator (take up 0.25 μL in 2 metal lamina, and touch surface). Apply 0.5 μL sample and dry under stream of cool air to avoid spot broadening. Repeat process as often as necessary for visualization of samples (see Table 1). Wet membrane with boric acid buffer before starting electrophoresis at 300 V for 20 min. For quantitation of guar or carob, wet application spot as well as membrane, before electrophoresis, with carbonate buffer instead of boric acid buffer, but use boric acid as cell buffer. This treatment aids migration.

Staining Procedures

For all electrophoretic procedures, use polystrol containers (10 × 13 × 8 cm) with covers.

(a) *Staining with Toluidine Blue and Methylene Blue.*—Dip membrane into ca 50 mL staining solution and gently shake in dye 20 s. Remove excess dye by rinsing in 5 portions of ca 40 mL tap water, each ca 20 s. For staining with Toluidine Blue, add 10 mL denaturated alcohol (90%) to last portion of water and rinse ca 20–30 s. Avoid prolonged washings because it removes all stain. Carrageenans, furcellaran, and agar change from blue to violet, which can also be useful for identification. Let these sheets dry in open air attached to glass plate.

(b) *Staining with Amido Black 10B.*—After electrophoresis, put membrane into solution of 5% trichloroacetic acid for 5 min. Stain as described above. Destain with 20 mL portions of methanol–acetic acid (9 + 1). Make sheet transparent by attaching to glass plate and heating 5 min at 105°C.

(c) *Staining with Fuchsin reagent.*—Incubate membrane in periodic acid solution exactly 5 min. Cautiously remove excess with 4 portions of ca 40 mL tap water, shaking 30–60 s each time. Plunge sheet into 30 mL colorless, filtered solution of Fuchsin reagent and hold until whole sheet is intense red (30 min to 10 h, depending on quality of reagent). Remove excess dye with 15 mL portions of 90% denatured alcohol, until membrane has become white or pink and substances are visible as red zones. For sheets kept in denatured alcohol, color keeps even for weeks. After quantitation, membrane can be made transparent for storage and documentation.

Isolating Gelatin

Heat 5–10 g sample 1 h in boiling water after addition of 20–40 mL water. Set pH to 3–4 with concentrated acetic acid, and centrifuge. If sample contains fat, remove upper layer (fat) carefully. Transfer liquid phase to 25 or 50 mL volumetric flask and dilute with water. Apply on membranes in position C (third application row of cell cover). Use carbonate buffer for

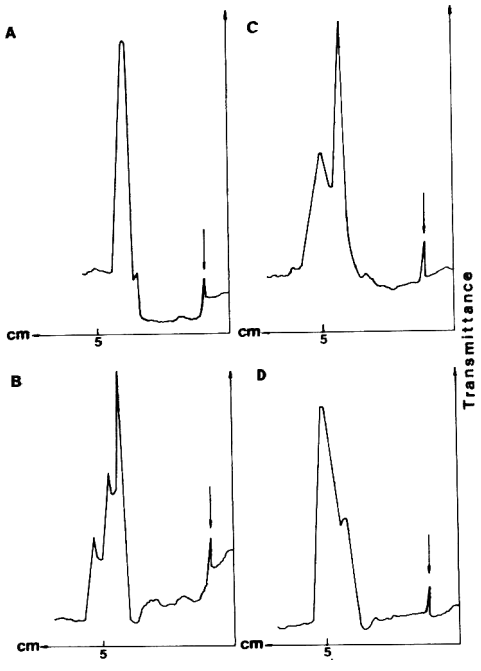


Figure 1. Scans of different types of carrageenans: (A), iota-type (pure); (B), kappa-type (pure); (C), mixture of 80% kappa + 20% lambda; (D), mixture of lambda + kappa + iota (1 + 1 + 1). Wavelength 550 nm, scan speed 10 mm/min.

electrophoresis and let run 15 min at 200 V. Stain with Amido Black 10B.

Procedure for Making Membrane Transparent

Remove denatured alcohol and add 20 mL methanol to remove water. Let stand 5 min. Place sheet on glass plate, pour ca 10 mL freshly prepared mixture of methanol–acetic acid over it, and let stand 60 s. Remove excess liquid and air bubbles with rubber policeman. Dry membrane attached to the glass plate 5 min at 105°C.

Identification Procedure

Samples usually can be identified using only 2 staining procedures: Toluidine Blue and Fuchsin reagent. To do this, apply to one sheet some of the standards with different migration behavior reacting with Toluidine Blue (Table 1) as well as different amounts of unidentified samples. Repeat by applying Fuchsin-sensitive substances (Table 1) and samples on a second sheet.

Quantitation

Wet membrane with water (for Toluidine Blue and Methylene Blue), methanol (Amido Black), or denatured alcohol (Fuchsin). Press between glass plates, avoiding air bubbles, and measure transmittance in TL-scanner at wavelength of 550 nm. Light path has to be reduced by slit blend (width 3.5 mm, length 6 mm); slit width: 0.01–0.05 mm, depending on color intensity of membrane background. Set XY-recorder to 0.5–0.05 mV/cm for signals of appropriate height. Use scan speed of 50 mm/min; then reduce to 10 mm/min to identify peak. Some special peak forms are given in Figure 1 (A–D).

Calculate detection limits as 2- or 3-fold average of peak heights in background within peak area, expressed as ng thickener.

Results and Discussion

In previous studies, we attempted to separate thickeners by using polyacrylamide gels, but migration was slowed by the narrow porous structures of the gels. Separation depends on the 3-dimensional structure of polysaccharides as well as their charges. Migration distances were short even when diluted polyacrylamide gels were used. In addition, the destaining procedure was lengthy, lasting up to 24 h because of the thickness of the gel. An alternative support material, agarose, is easier to handle and gives better migration distances. However, on this gel, some thickeners migrate in a sharp line, while others migrate as broad zones. Agarose gel separation of some thickeners is shown in Figure 2.

Finally, cellulose acetate membranes were chosen to avoid retention caused by 3-dimensional structures of thickeners. The separation depends only on the charged groups like sulfuric esters or uronic acids. The use of boric acid

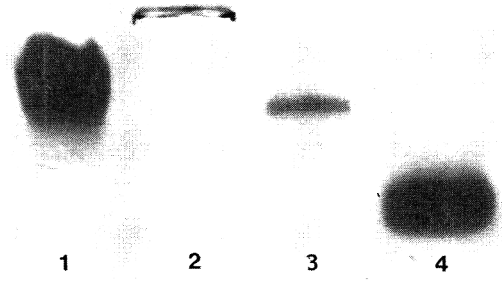


Figure 2. Electrophoretic separation of thickeners on agarose gel at 100 V for 20 min, using boric acid buffer, stained by Fuchsin: 1, pectin, 10 μL, 0.5% solution; 2, soluble starch, 10 μL, 0.06% solution; 3, gum arabic, 10 μL, 0.6% solution; 4, alginic acid, 10 μL, 1% solution.

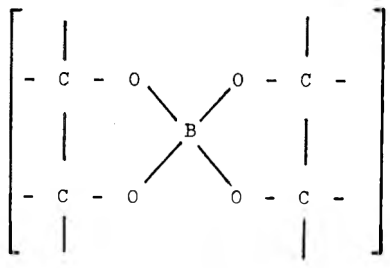


Figure 3. Complex of boric acid buffer formed with uncharged thickeners.

buffer to form a complex (Figure 3) with uncharged thickeners, like guar and carob, allows anodic migration of all polysaccharides.

As already mentioned, identification of thickeners depends on, first, evidence of migration behavior (first criterion) as shown in Figures 4 and 5 for thickeners stained by Fuchsin and Toluidine Blue. Migration distance of all thickeners compared with gum arabic is given in Table 2. Second, special shapes of electrophoretic zones are demonstrated with different types of carrageenans (Figure 6), carboxy methyl cellulose, and pectin (Figure 5). As shown in Figure 5, all types of carrageenans (kappa, iota, lambda) can be distinguished by their zone shape. Ability to be stained is the third criterion (see Table 1). Some polysaccharides which are stained by Toluidine Blue, like carrageenans, furcellaran, and agar-agar, change color from blue to violet in contact with dilute alcohol. This can be used as an additional identification factor. Combining these criteria, we can recognize all

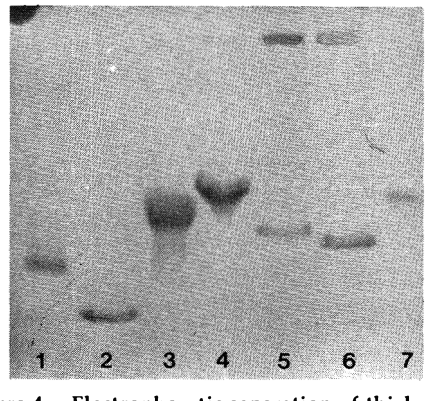


Figure 4. Electrophoretic separation of thickeners on cellulose acetate membrane at 300 V for 20 min, stained with Fuchsin: 1, 254 ng gum arabic; 2, 1250 ng Na-alginate; 3, 3750 ng gum ghatti; 4, 1250 ng tragacanth; 5, 300 ng carob; 6, 150 ng guar; 7, 1250 ng starch.

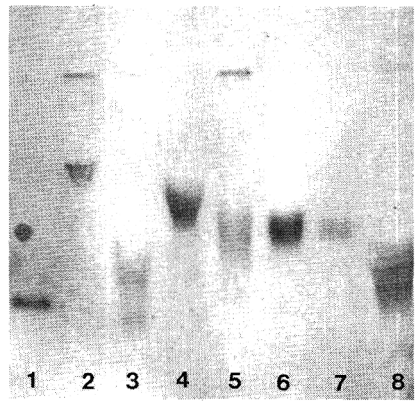


Figure 5. Electrophoretic separation of thickeners on cellulose acetate membrane at 300 V for 20 min, stained with Toluidine Blue: 1, 250 ng alginic acid; 2, 1875 ng tragacanth; 3, 20 000 ng agar-agar; 4, 1562.5 ng pectin; 5, 500 ng carboxy methyl cellulose; 6, 125 ng propylene glycol alginate; 7, 625 ng gum arabic; 8, 375 ng kappa-carrageenan.

usual types of thickeners, as well as gelatin.

The only difficulty was separation of the galactomannans guar and carob (compare spots 5 and 6 in Figure 4). Similarity of chemical composition and constituents as well as their uncharged form makes electrophoretic separation impossible. Distinction is possible only if samples contain either one or the other. In such samples, a microscopic identification of guar and

Table 2. Migration of thickeners relative to gum arabic

Thickener	Schiff's reagent stain	Toluidine Blue stain
Agar–agar	—	0.64/1.22[a]
Alginic acid	1.34	1.34
Ca-Alginate	1.2	1.2
Carob	0.89	—
kappa-Carrageenan	—	1.2/1.42[a]
lambda-Carrageenan	—	1.48
iota-Carrageenan	—	1.3/1.6[a]
Guar	0.95	—
Carboxy methyl cellulose	—	1.04
Gum arabic	1.0	1.0
Gum ghatti	0.81	0.81
Gum karaya	—	1.0
K-Alginate	1.2	1.2
Na-Alginate	1.2	1.2
Pectin	0.8	0.8
Propylene glycol alginate	1.07	1.07
Starch	0.72	—
Tragacanth	0.69	0.69

[a] Substance gives 2 peaks.

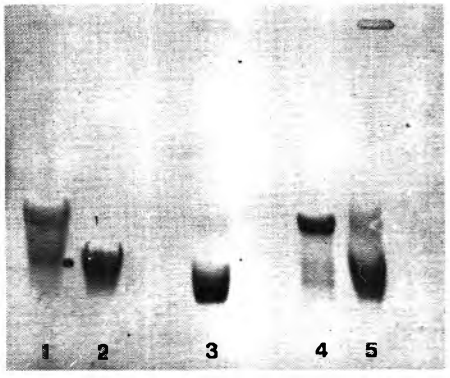


Figure 6. Electrophoretic separation of different types and mixtures of carrageenans on cellulose acetate membrane at 300 V for 10 min, stained with Toluidine Blue: 1, 750 ng kappa-carrageenan; 2, 376 ng lambda-carrageenan; 3, 750 ng iota-carrageenan; 4, 600 ng (80%) kappa + 175 ng (20%) lambda-carrageenan; 5, 350 ng (40%) kappa + 175 ng (20%) lambda + 350 ng (40%) iota-carrageenan.

carob should precede quantitation by electrophoresis.

Another problem is the fact that relatively high amounts of these thickeners are retained on the application spot. Only a portion migrates, so electrophoretic quantitation gives low results. Soaking the cellulose acetate membrane in carbonate buffer before electrophoresis enables complete migration of the applied sample and should be used for quantitation studies.

Some different alginates (alginic acid, Na-, Ca-, K-alginate, and propylene glycol alginate) can be separated by migration behavior (compare spots 1 and 6 in Figure 5), but K-, Na-, and Ca-alginate cannot (compare Table 2). Pectins can be classified on the basis of their low or high amount of esters and different contents of amidated groups. The 2 types of gelatin (A and B) show different migration behavior. Type A, produced by an acidic hydrolysis, has its isoelectric point (IEP) at pH 8–9. The alkaline digestion gives type B with an IEP at pH 4.8–5. With carbonate buffer, type A migrates less than B. All other concomitantly isolated proteins are clearly separated and distinguishable. Only very small differences in migration behavior were observed for the different types of modified starch. Thus, identification by this method is impossible.

We have analyzed all common types of thickeners produced by different companies. In all cases, pure thickeners from the same type showed the same electrophoretic behavior regardless of the producer. We have also checked the possibility of degradation of some thickeners. After treatment under the usual conditions of sterilization, no change in migration behavior or degradation was observed for carob, guar, or alginic acid compared with untreated samples. No enzymatic degradation by α-amylase was observed after 3 h treatment of guar, carob, or tragacanth. However, migration after 48 h treatment reflected a partial destruction of the α-1,4-bonds.

Incompleteness of starch degradation does not interfere with separation. Therefore, an enzymatic treatment should precede electrophoresis in samples rich in starch, like puddings, to avoid problems during resolution of the precipitated polysaccharides.

The great advantage of the presented method is the variability in application volume. Samples

Table 3. Limits of detection (ng) for different thickeners [a]

	Amido Black		Schiff's reagent		Toluidine Blue	
Thickener	2σ	3σ	2σ	3σ	2σ	3σ
Gelatin	180	280	—	—	—	—
Agar–agar	—	—	—	—	11 900	13 900
Carrageenan	—	—	—	—	40	95
Carob	—	—	106	120	—	—
Carboxy methyl cellulose	—	—	—	—	160	200
Guar	—	—	40	60	—	—
Gum arabic	—	—	10	20	120	400
Gum ghatti	—	—	120	260	1400	1700
Na-Alginate	—	—	800	900	20	30
Pectin	—	—	560	920	290	295
Soluble starch	—	—	420	480	—	—
Tragacanth	—	—	90	140	700	1000

[a] 2σ and 3σ = 2× and 3× background noise, expressed as ng substance.

Table 4. Quantitative determination of thickeners in food

Sample No.	Product	Thickener found, %		Rec., %
1	Vanilla pudding cream I	2.1	carrageenan	90.1
2	Vanilla pudding cream II	4.06	carrageenan	92.5
		0.43	carob	114.7
3	Pudding	0.94	carob	86
		0.18	Na-alginate	87
4	Babyfood, milk-based I	0.74	guar	98.6
5	Babyfood, milk-based II	0.46	guar	92
6	Gelle sugar fruits I	1.75	pectin	101.7
7	Gelle sugar fruits II	0.95	agar	92.2
8	Ice cream I	0.16	carob	100
		0.016	kappa-carrageenan	100
9	Ice cream II	0.19	carob	95
		0.1	kappa-carrageenan	100
10	Ice cream III	0.18	carob	105.8
		0.71	gelatin	118
11	Ketchup	0.44	guar	88
12	Cream stabilizer	2.8	kappa-carrageenan	93.3
		3.4	carob	68

of 0.25–2.5 μL, containing 25–25 000 ng thickeners, can be separated on the cellulose acetate membrane (see Table 1). Reproducibility of application is ±6%, calculated as the standard deviation of the peak heights of 8 applications of the same amount of substance on several sheets. Reproducibility of measurement, using the Zeiss scanner, is ±0.3%. As mentioned earlier, the isolation process of the thickeners must be selected in consideration of the total content of the product. In absence of one or more components, the process can be shortened. Limits of detection are given in Table 3.

Removal of fat and dyes with dioxane also has the advantage of splitting complexes between proteins and thickeners, as observed with carrageenans (2, 20). Cleavage of such a complex is necessary for quantitative isolation of the thickener. During precipitation of proteins with trichloroacetic acid, alginates might coprecipitate, as reported by some authors (1, 20, 22). Addition of urea dissolves proteins before their precipitation and should be done to avoid loss of some types of thickeners (agar, alginates, methyl cellulose) (2, 20).

To test the presented method, we analyzed different types of commercial samples (Table 4), supplied by different producers. The composition of the thickeners was given to us after tests were completed. With the exception of Samples 6, 7, and 10, the complete isolation procedure was applied. For Samples 6 and 7, dissolving in water and precipitation of polysaccharides by absolute ethanol was sufficient. When isolating thickeners from ketchup (Sample 11), starch degradation proved unnecessary. Since the recovery for guar of Sample 11 was 88% compared with 98.6% and 92% for Samples 4 and 5, where starch degradation was used, α-amylase treatment under our conditions did not influence results.

Recovery of carrageenans is between 90.1 and 100%. These values compare well with the findings of Glück and Thier (20) who recovered 90%. The good recoveries reflect the positive influence of dioxane extraction, as well as the protein-removing step, which supports splitting of complexes between carrageenans and proteins.

For agar, some difficulty in quantitation has been observed. When agar, dissolved in acid and neutralized, was subjected to electrophoresis, 2 zones were observed. Precipitation of the polysaccharides by ethanol results in the loss of one of these zones. This might be caused by the 2-component nature of agar, which consists of uncharged agaropectin and the charged agarose. Therefore, for quantitation, an acid-treated, ethanol-precipitated standard must be used.

The reliability of the isolation process has been determined using special breads containing guar. Recovery from 8 breads containing 0.35% guar was 95.1 ± 4.7%, and at a concentration of 0.17% guar, 95.9 ± 2.1%. We applied our method to about 300 different samples, mainly sauces of canned fish, instant drinks, and puddings, as well as condensed milk, gingerbread, salad dressings and other creams based on mayonnaise, cheese powders for fondues, liquids of canned caviar, and stabilizers for home-made jams and sausage production. All samples showed good qualitative and quantitative results,

in spite of the very different nature of the examined products.

Acknowledgment

We are grateful to the Forschungsförderungsfonds der gewerblichen Wirtschaft for sponsoring this work during 1978 and 1979.

References

(1) Schweizernsches Lebensmittelbuch Methoden fur die Unter-suchung und Beurteilung von Lebensmitteln und Gebrauchsgegenstanden, II, Teil, Kap. 40: Gelier-u. Vendickungsmittel 5, Auflage, Bonn, 1977
(2) Morley, R. G., Phillips, G. O., & Power, D. M. (1972) *Analyst* **97,** 315-319
(3) Letzig, E. (1955) *Dtsch. Lebensm. Rundsch.* **2,** 41-47
(4) Klostermeyer, H., Popp, U., & Reimerdes, E. H. (1975) *Z. Lebensm. Unters. Forsch.* **158,** 73-75
(5) Friese, P. (1980) *Z. Anal. Chem.* **301,** 389-397
(6) Grau, R., & Schweiger, A. (1962/1963) *Z. Lebensm. Unters. Forsch.* **119,** 210-216
(7) Sulser, H. (1957) *Mitt. Geb. Lebensmittelunters. Hyg.* **48,** 19-29
(8) Friese, P. (1980) *Z. Anal. Chem.* **303,** 279-288
(9) Schmolck, W., & Mergenthaler, E. (1973) *Z. Lebensm. Urters, Forsch.* **152,** 263-273
(10) Mergenthaler, E., & Scherz, H. (1976) *Z. Lebensm. Unters. Forsch.* **162,** 25-29
(11) Mergenthaler, E., & Scherz, H. (1976) *Z. Lebensm. Unters. Forsch.* **162,** 159-162
(12) Mergenthaler, E., & Scherz, H. (1978) *Z. Lebensm. Unters. Forsch.* **166,** 225-227
(13) Untze, W. (1977) *Lebensmittelchemie Ger. Chem.* **31,** 112-116
(14) Mergenthaler, E., & Schmolck, W. (1974) *Z. Lebensm. Unters. Forsch.* **155,** 193-202
(15) Padmoyo, M., & Miserez A. (1967) *Mitt. Geb. Lebensmitteiunters. Hyg.* **58,** 31-49
(16) Hidalgo, J. P., & Hansen, M. T. (1968) *J. Food Sci.* **33,** 7-11
(17) Buckee, G. K. L., Dolezil, Forrest, I. S., & Hickman, E. (1976) *J. Inst. Brew. London* **82,** 209-21
(18) Do, J. Y., Ioannou, J., & Haard, N. F. (1971), *J. Food Sci.* **36,** 1137-1138
(19) Huntziker, H. R., & Miserez, A. (1930) *Mitt. Geb. Lebensmittelunters. Hyg.* **71,** 87-94
(20) Glück, U., & Thier, H. P. (1980) *Z. Lebensm. Unters. Forsch.* **170,** 272-280
(21) Padmoyo, M., & Miserez, A. (1965) *Mitt. Geb. Lebensmittelunters. Hyg.* **56,** 110-125
(22) Schmolck, W., & Mergenthaler, W. (1973) *Z. Lebensm. Unters. Forsch.* **152,** 87-99
(23) Ebermann, R., & Barna, J. (1972) *Z. Lebensm. Unters. Forsch.* **148,** 391-393

DRUGS

Reverse Phase High Performance Liquid Chromatographic Determination of Phenprocoumon in Tablets

WALTER F. SCHMIDT
Food and Drug Administration, 60 Eighth St, Atlanta, GA 30309

A high performance liquid chromatographic procedure has been developed for the assay of phenprocoumon in tablets. In comparison to the present official USP assay procedure, it is equivalent in precision and accuracy and is faster and more specific. A mobile phase consisting of a 1% solution of acetic acid in acetonitrile-water (4 + 3) separates phenprocoumon from warfarin internal standard on a 6 μm octadecylsilane (ODS) column with UV detection at 311 nm. The method enables the concurrent determination of phenprocoumon and possible contaminants such as salicylic acid.

Phenprocoumon, 4-hydroxy-3-(1-phenylpropyl)-2*H*-1-benzopyran-2-one, is a long-acting anticoagulant (1). Official USP XX assay methodology (2) for phenprocoumon in tablets requires multiple extractions followed by a UV spectrophotometric determination. The content uniformity procedure is essentially the same except that no extractions are required to separate the drug from the other tablet excipients.

Gas chromatography (GC) and high performance liquid chromatography (HPLC) have been applied to the analysis of phenprocoumon. The GC methods (3–5) involve derivative formation to improve resolution and sensitivity. The derivatives (trimethylsilyl, acetates, and methyl ethers) are detected by flame ionization and electron capture detectors. The procedures were designed for the assay of phenprocoumon in biological samples rather than in dosage forms.

Both normal and reverse phase HPLC methods have been reported (6) for the separation of phenprocoumon from other chemically related coumarin anticoagulants and phenprocoumon decomposition products. None of these HPLC procedures was applied to the assay of the drug and/or its decomposition products in dosage forms. In this paper, a reverse phase HPLC procedure is reported for phenprocoumon in tablets as an improved alternative to the official USP XX procedure. The HPLC method will allow the concurrent determination of phenprocoumon and possible contaminants such as salicylic acid. Assay and content uniformity results for phenprocoumon tablets by the HPLC method are compared to those by the USP XX methodology.

Received July 27, 1981. Accepted November 3, 1981.

METHOD

Apparatus

(a) *Liquid chromatograph.*—With variable wavelength detector (Varian Model 8500 with "Vari-Chrom" detector, Varian Associates, Palo Alto, CA 94303, or equivalent).

(b) *Loop type injector.*—10 μL loop (Rheodyne, Berkeley, CA 94710, or equivalent).

(c) *HPLC column.*—Stainless steel, 25 cm × 4 mm id; packed with octadecylsilane (ODS) 6 μm silica particles (Zorbax ODS column purchased prepacked from E.I. du Pont de Nemours and Co., Wilmington, DE 19898, or equivalent).

(d) *Filter paper.*—Pore size 0.7 μm (Whatman GF/F, Whatman, Inc., Clifton, NJ 07014, or equivalent).

(e) *Ultrasonic generator.*—(Lenape Model 830, Lenape Equipment Co., Plainfield, NJ, or equivalent).

Reagents

(a) *Mobile phase.*—Combine 400 mL acetonitrile (UV-grade, Burdick & Jackson Labs, Muskegon, MI 49442, or equivalent), 300 mL double-distilled water, and 7 mL acetic acid. Mix, pass through 0.7 μm filter paper, and degas.

(b) *Internal standard solution.*—Dissolve ca 25 mg Warfarin Sodium (USP Reference Standard or other suitable grade) in 25.0 mL mobile phase.

(c) *Standard solution.*—Dissolve ca 25 mg USP Reference Standard Phenprocoumon, accurately weighed, in 25.0 mL mobile phase. Transfer 3.0 mL standard solution into a 100.0 mL flask. Add 3.0 mL internal standard solution and dilute to volume with mobile phase.

Determination

Determine average weight of tablets and reduce to fine powder. Accurately weigh pow-

0004-5756/82/6503-0753-03$01.00

Table 1. Comparison of HPLC and USP XX assay for phenprocoumon in tablets (replicate determinations on the same composite) [a]

	HPLC, mg/tab.	% Declared	USP, XX, mg/tab.	% Declared
	3.01	100.3	2.98	99.3
	3.00	100.0	2.95	98.3
	3.04	101.3	2.97	99.0
	3.01	100.3	2.93	97.7
	3.01	100.3	2.99	99.7
	3.03	101.0	3.01	100.3
	2.99	99.7	2.95	98.3
	3.00	100.0	3.01	100.3
	2.96	98.7	2.93	97.7
	3.00	100.0	2.97	99.0
Mean	3.005		2.969	
SD	0.022		0.029	
CV, %	0.72		0.98	
F-ratio	1.81			

[a] At the 95% confidence level, the critical value for the *F*-test, when $DF_1 = 9$ and $DF_2 = 9$, is 3.18. Difference in mean values = 0.036 ± 0.084 (18 degrees of freedom, 95% confidence level).

dered sample equivalent to ca 3 mg phenprocoumon and transfer to a 100 mL flask. Add 3.0 mL internal standard solution to flask and dilute to ca 25 mL with mobile phase. Agitate solution ultrasonically for 10–15 min, dilute to volume with mobile phase, mix, and pass 5–10 mL through 0.7 μm filter paper. Set UV detection wavelength at 311 nm, and adjust flow rate to 1 mL/min. Inject 10 μL sample filtrate and 10 μL standard solution into liquid chromatograph. Obtain chromatograms and determine peak response by peak height or peak area. Calculate μg phenprocoumon in portion of sample taken by the formula

$$(R_u/R_s) \times C$$

where R_u and R_s are the ratio of peak response of phenprocoumon to internal standard for sample and standard phenprocoumon, respectively, and C is the concentration of phenprocoumon standard in μg/mL.

Results and Discussion

The HPLC assay described here was validated by comparing it to the USP XX methodology. Twenty replicate determinations were performed on the same phenprocoumon tablet composite, 10 by the USP XX procedure and 10 by the HPLC procedure (Table 1). No significant difference was found (95% confidence interval) between the mean USP XX assay value of 2.97 mg/tablet and the mean HPLC assay value of 3.00 mg/tablet. The coefficients of variation (CV) were 0.98% by the USP XX procedure and 0.72% by the HPLC procedure. At the 95% confidence level, the difference in precision was not significant ($F = 1.81$; critical value = 3.18).

Twenty individual tablets from a different sample batch were then assayed for phenprocoumon, 10 by the USP XX content uniformity procedure and 10 by the HPLC assay procedure (Table 2). No significant difference was found (95% confidence interval) between the mean USP XX content uniformity assay value of 3.09 mg/tablet and the mean HPLC content uniformity assay value of 3.12 mg/tablet. The CV was 2.22% by the USP XX procedure and 1.57% by the HPLC procedure. The value of F was 1.97. Therefore, in the determination of content uniformity the HPLC procedure shows no significant difference in variance from the USP procedure at the 95% confidence level.

Table 2. Comparison of content uniformity determinations of phenprocoumon in tablets by HPLC and USP XX procedures [a]

Tablet No.	HPLC, mg/tab.	% Declared	Tablet No.	USP XX, mg/tab.	% Declared
1	3.15	105.0	11	3.07	102.3
2	3.12	104.0	12	3.13	104.3
3	3.18	106.0	13	3.14	109.7
4	3.13	104.3	14	3.14	104.7
5	3.17	105.7	15	3.09	103.0
6	3.04	101.3	16	3.05	101.7
7	3.14	104.7	17	3.04	101.3
8	3.04	101.3	18	2.96	98.7
9	3.09	103.0	19	3.21	107.0
10	3.14	104.7	20	3.08	102.7
Mean	3.120			3.091	
SD	0.049			0.069	
CV, %	1.57			2.22	
F-ratio	1.97				

[a] At the 95% confidence level, the critical value for the *F*-test, when $DF_1 = 9$ and $DF_2 = 9$, is 3.18. Difference in mean values = 0.029 ± 0.032 (18 degrees of freedom, 95% confidence level).

Table 3. Recovery results from HPLC determination of standard phenprocoumon added to a synthetic mix

Added, mg	Found, mg	% Rec.
2.484	2.458	99.0
2.625	2.629	100.2
2.892	2.894	100.1
3.032	2.970	98.0
3.523	3.528	100.1
3.612	3.580	99.1
Mean		99.4
SD		0.88
CV, %		0.88

Recovery analysis further validates the HPLC procedure. Six different levels of phenprocoumon were added to a placebo formulation and determined by the HPLC procedure (Table 3). The mean recovery of phenprocoumon was 99.4%, and CV was 0.88%.

Linear regression analysis (n = 4) of phenprocoumon (peak heights vs concentration; 0–40 μg/mL) gave a correlation coefficient of 0.9998. The detection limit for phenprocoumon at signal/noise ratio of 2 was 0.01 μg per injection.

The reproducibility of the chromatographic system was determined by injecting six 20 μL portions of phenprocoumon standard solution and comparing the ratio of phenprocoumon and warfarin peak heights. The CV of 6 injections was ±0.46%. The resolution factor between the 2 peaks was 2.7. Each of the 2 peaks had a tailing factor of less than 1.2.

The USP XX compendial monograph for phenprocoumon lists salicylic acid as a potential contaminant (2). UV detection at 311 nm allows separation and detection of both phenprocoumon and salicylic acid (Figure 1).

The HPLC procedure is therefore comparable to the USP XX assay and content uniformity procedures in accuracy and precision. In addition, it is more rapid and specific than the official procedure. This procedure also allows the determination of possible contaminants such as salicyclic acid.

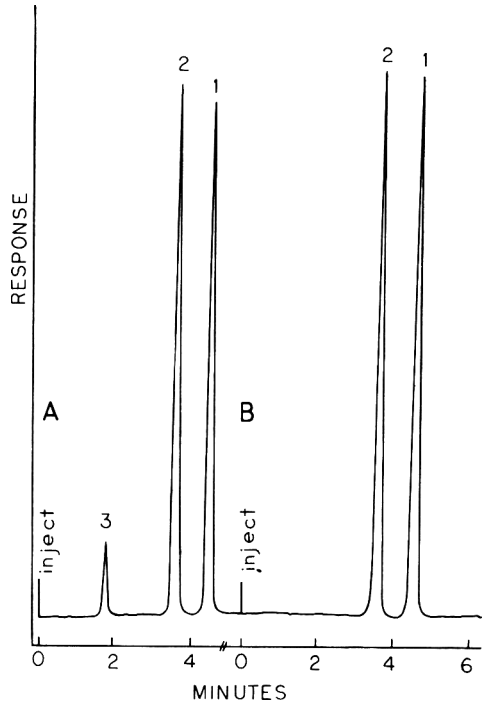


Figure 1. Chromatograms of A, standard mixture: (1) phenprocoumon, (2) warfarin, and (3) salicylic acid, a potential contaminant; B, sample: (1) phenprocoumon and (2) warfarin internal standard.

Acknowledgments

The author thanks Stephen Sherkin, Scientific Coordinator, FDA, Rockville, MD, for his statistical analysis and James T. Stewart of the University of Georgia, Science Advisor, for editorial assistance.

References

(1) Lewis, A. J. (Ed.) (1979) *Modern Drug Encyclopedia*, 15th Ed., York Medical Press, New York, NY, p. 752
(2) *U.S. Pharmacopeia* (1980) 20th Rev., U.S. Pharmacopeial Convention, Rockville, MD, p. 614
(3) Deckert, F. W. (1972) *J. Chromatogr.* **64**, 355–357
(4) Deckert, F. W. (1972) *J. Chromatogr.* **69**, 201–203
(5) Midha, K. K., Hubbard, J. W., Cooper, J. K., & McGilveray, I. J. (1976) *J. Pharm. Sci.* **65**, 387–391
(6) VanHaelen-Fastre, R., & VanHaelen, M. (1976) *J. Chromatogr.* **129**, 397–402

Liquid Chromatographic Identification of *cis*- and *trans*-Cinnamoylcocaine in Illicit Cocaine

F. TAYLOR NOGGLE, JR and C. RANDALL CLARK[1]
Alabama Department of Forensic Sciences, Wire Rd, Auburn, AL 36830

A liquid chromatographic procedure is described for the analysis of illicit cocaine samples. The isomeric *cis*- and *trans*-cinnamoylcocaines are separated from cocaine, benzoylecgonine, and benzoic acid in a reverse phase isocratic system with dual wavelength detection at 254 and 280 nm. Specific identification of cocaine and the cinnamoylcocaines is accomplished by ultraviolet spectrophotometry and mass spectrometry of the chromatographic effluent.

Cocaine is one of the more frequently encountered drugs in forensic laboratories. In addition to its high potential for abuse, cocaine has legitimate medicinal value in several therapeutic situations. Questions have arisen in court proceedings (1, 2) concerning the specificity of instrumental and chromatographic techniques for differentiating the diastereoisomers and enantiomers of cocaine. High pressure liquid chromatography (HPLC) is an effective method for differentiating the diastereoisomers of cocaine (3). However, determination of the enantiomeric composition is more difficult. Allen et al. (4) described procedures in which cocaine is reacted with heavy metal salts, gold chloride and platinic chloride, to provide the unequivocal assignment of the enantiomeric form of cocaine, and also infrared spectrophotometry based on differences in the spectra of a racemic mixture of cocaines and the pure enantiomers.

The majority of illicit cocaine is believed to be produced by extraction and purification of the South American coca plant which contains the alkaloids (−)-cocaine, cinnamoylcocaine, the truxillines, and tropacocaine (5). Pharmaceutical grade cocaine is prepared by the hydrolysis of cocaine and other alkaloids to ecgonine, followed by double esterification (methylation and benzoylation) to yield cocaine. Moore (6) identified the presence of *cis*- and *trans*-cinnamoylcocaine in over 50% of illicit cocaine samples analyzed, usually in concentrations of 1% or less of the amount of cocaine. In addition to the cinnamoylcocaines, illicit cocaine samples also contain varying quantities of benzoylecgonine, ecgonine, methylecgonine, and benzoic acid (7).

The detection of the cinnamoylcocaines in illicit cocaine is of importance to the forensic chemist for several reasons: (1) to confirm the logical presumption that the cocaine was purified from the coca plant and therefore (−)-cocaine; (2) to determine the procedure used in manufacturing the cocaine; (3) to compare with other cocaine samples to determine if they are of common origin; (4) to determine the accuracy of quantitation by ultraviolet (UV) spectrophotometry using the 275 nm peak of cocaine.

Most procedures for separation and detection of minor alkaloids in cocaine specify gas chromatography with flame ionization detection (GC-FID). Moore (6) used this procedure to identify *cis*- and *trans*-cinnamoylcocaine in illicit cocaine. Another procedure by Moore (8) specifies GC-FID detection of ecgonine and benzoylecgonine after silylation with *N,O*-bis-(trimethylsilyl)acetamide (BSA). BSA is also useful in the detection of methylecgonine in illicit cocaine (7). Lukaszewski and Jeffery (9) found that methylecgonidine is the compound most consistently encountered during gas chromatographic/mass spectrometric identification of cocaine. Methylecgonidine is formed as a result of elimination of benzoic acid from cocaine in the heated injection port of the gas chromatograph.

Liquid chromatography is an especially useful technique for separation of polar, nonvolatile, or thermally unstable compounds such as cocaine, benzoylecgonine, and related compounds. Various authors have described the detection and separation of cocaine and benzoylecgonine in urine (10) and human plasma (11). Lurie (12) described an ion-pair partition method for separating various local anesthetics and cocaine, but none of the minor alkaloids related to cocaine were included in the study. In this paper, the usefulness of HPLC is demonstrated for separating and identifying cocaine, benzoylecgonine, benzoic acid, and *cis*- and *trans*-cinnamoylcocaine in illicit cocaine, using a reverse phase isocratic system and dual wavelength UV detection.

Received August 13, 1981. Accepted December 20, 1981.
[1] Auburn University School of Pharmacy, Division of Medicinal Chemistry, Department of Pharmacal Sciences, Auburn University, AL 36849.

0004-5756/82/6503-0756-06$01.00

Experimental

Reagents and Chemicals

Cocaine hydrochloride (USP, Merck & Co., Inc., Rahway, NJ), benzoic acid and HPLC grade methanol (Fisher Scientific Co., Fair Lawn, NJ) were used. *trans*-Cinnamoylcocaine base and benzoylecgonine hydrochloride were obtained from the Drug Enforcement Administration, Special Testing and Research Laboratory, McLean, VA. Illicit cocaine samples were submitted to the Alabama Department of Forensic Sciences by various law enforcement agencies in Alabama.

The pH 3.0 phosphate buffer was prepared by mixing 9.2 g monobasic sodium phosphate (NaH_2PO_4) in 1 L double-distilled water and adjusting the pH to 3.0 with 2N H_3PO_4.

Apparatus

Ultraviolet spectra were recorded on a Hitachi 100-80 spectrophotometer (Tokyo, Japan); mass spectra were recorded on a Dupont 490B mass spectrometer (E.I. du Pont de Nemours and Co., Inc., Wilmington, DE 19818) equipped with a Finnigan INCOS data system (Sunnyvale, CA 94086). A Beckman Model 3500 pH meter was used for all pH measurements. The liquid chromatograph consisted of a Waters Associates (Milford, MA 01757) Model 6000A pump, Model U6K injector, Model 440 UV detector with dual wavelength accessory operated at 254 and 280 nm, and Houston Instrument (Austin, TX 78753) OmniScribe dual pen recorder.

Chromatographic Procedures

All separations were carried out on a 30 cm × 3.9 mm id μBondapak C_{18} column (Waters Associates) with a pH 3.0 phosphate buffer–HPLC grade methanol (2 + 1) mobile phase. The analytical column was preceded by a 7 cm × 2.1 mm id guard column dry-packed with CO:PELL ODS (Whatman Inc., Clifton, NJ). Mobile phase flow rate was 2.0 mL/min and the UV detector was operated at 0.2 AUFS. Sample solutions were prepared in HPLC grade methanol and all separations were made at ambient temperature.

Mass Spectrometry

The peaks for cocaine and *cis*- and *trans*-cinnamoylcocaine were collected from the chromatographic effluent. Individual solutions were made basic with dilute ammonium hydroxide and were extracted with methylene chloride. The extract was evaporated to dryness and the residue was dissolved in a small amount of methanol. The methanol solution was taken up in the tip of a capillary tube and evaporated to dryness. The residue on the tip of the capillary tube was analyzed by electron impact mass spectrometry (70 eV) via the solid probe.

Results and Discussion

The liquid chromatographic analysis of illicit cocaine samples revealed the presence of several UV-absorbing compounds. The reverse phase (C_{18}) separation of these compounds was maximized by using an isocratic solvent system of pH 3.0 phosphate buffer–methanol (2 + 1). Separations obtained from 2 representative samples are shown in Figure 1. The highly acidic mobile phase produces good resolution and sufficient solute retention. Under these conditions, cocaine (pKa 8.5) and other substituted tropane alkaloids should exist exclusively as the protonated amine. The formation of more hydrophobic ion-pairs by the addition of 1-heptanesulfonic acid to the mobile phase did not offer any advantages over the hydrophilic phosphate counter ion.

Cocaine hydrolysis products such as benzoylecgonine, methylecgonine, ecgonine, and benzoic acid are often observed in illicit cocaine samples along with cocaine and the minor tropane-type alkaloids (Figure 2). By comparison with available standards, peaks 1, 2, 3, and 5 in Figure 1 (A and B) matched the elution times for benzoylecgonine, cocaine, benzoic acid, and *trans*-cinnamoylcocaine, respectively. Methylecgonine and ecgonine contain no strong chromophoric groups and are essentially transparent at 254 and 280 nm. Thus, no peaks for these compounds were expected.

Further proof of the identity of peaks 1, 2, 3, and 5 was obtained from a comparison of the ratio of absorbances at 254 and 280 nm (A254/A280). The peak height ratios from the 254 and 280 nm detectors were determined for the standards and the components of the illicit cocaine samples. The results of this study (Table 1) further confirm the identity of peaks 1, 2, 3, and 5. The ratios obtained from the standards were identical to those of the sample peaks. Samples containing *trans*-cinnamoylcocaine usually contain the *cis*-isomer (6), thus peak 4 in Figure 1 (A and B) was suspected to be *cis*-cinnamoylcocaine. In an attempt to locate the peaks for the isomeric cinnamoylcocaines, a sample of illicit cocaine was subjected to mild oxidation with a solution of dilute potassium permanganate. The results of this experiment are shown in Figure 3. The most striking change in the oxidized sample

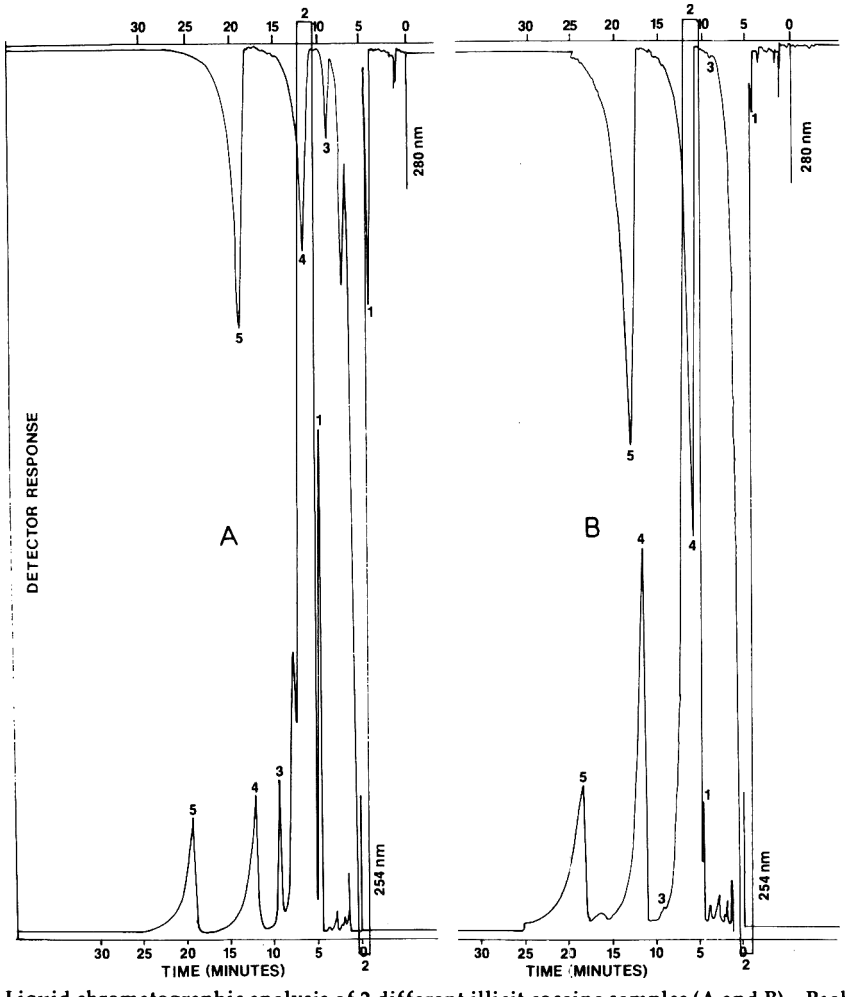


Figure 1. Liquid chromatographic analysis of 2 different illicit cocaine samples (A and B). Peaks: 1, benzoylecgonine; 2, cocaine; 3, benzoic acid; 4, *cis*-cinnamoylcocaine; 5, *trans*-cinnamoylcocaine. See text for chromatographic conditions.

is the absence of peaks 4 and 5. Olefinic double bonds such as those found in the cinnamoyl group are particularly susceptible to oxidation by potassium permanganate. The more polar oxidation products would be expected to elute earlier than the cinnamoylcocaine isomers. The fact that both peaks 4 and 5 represent compounds easily oxidized by potassium permanganate

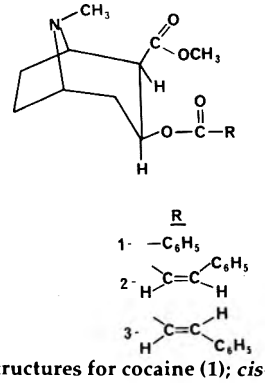


Figure 2. Structures for cocaine (1); *cis*-cinnamoylcocaine (2); and *trans*-cinnamoylcocaine (3).

Table 1. Retention and absorbance data for components of illicit cocaine samples

Peak No.[a]	Compound	Rel. retention time	A254/A280
1	benzoylecgonine	0.49	1.95
2	cocaine	0.65	1.95
3	benzoic acid	1.00 (18.2 mL)	1.58
4	*cis*-cinnamoylcocaine	1.30	0.84
5	*trans*-cinnamoylcocaine	2.07	0.37

[a] See Figure 1.

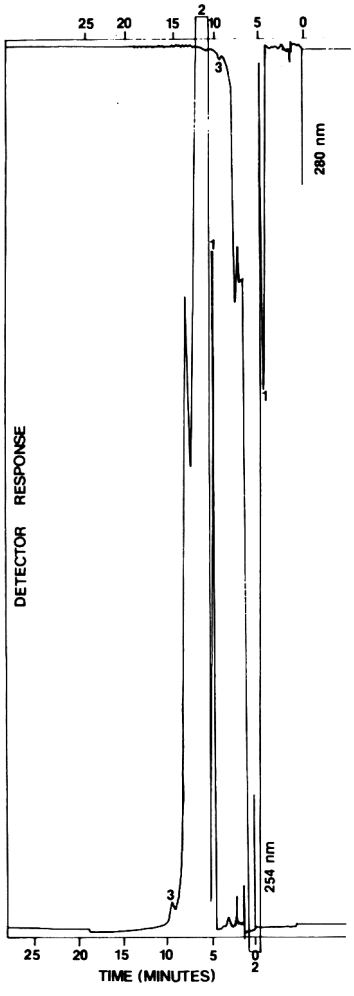


Figure 3. Liquid chromatographic analysis of the illicit cocaine sample in Figure 1A following treatment with dilute potassium permanganate. See Figure 1 for peak identification.

lends support to their tentative assignment as *cis*- and *trans*-cinnamoylcocaine.

The chromatographic effluent corresponding to peaks 4 and 5 in Figures 1A and 1B was collected and the UV absorption spectrum for each solution was recorded from 350 to 220 nm. The UV spectrum (Figure 4) for peak 5 in Figure 1A, which corresponded in retention time and absorption ratio with known *trans*-cinnamoylcocaine, was compared with the spectrum for known *trans*-cinnamoylcocaine and was identical. The UV spectrum (Figure 5) of the suspected *cis*-cinnamoylcocaine peak was shifted from a maximum absorbance of 280 nm for *trans*-cinnamoylcocaine to 275 nm and was of lesser intensity. The A254/A280 ratio (Table 1) for

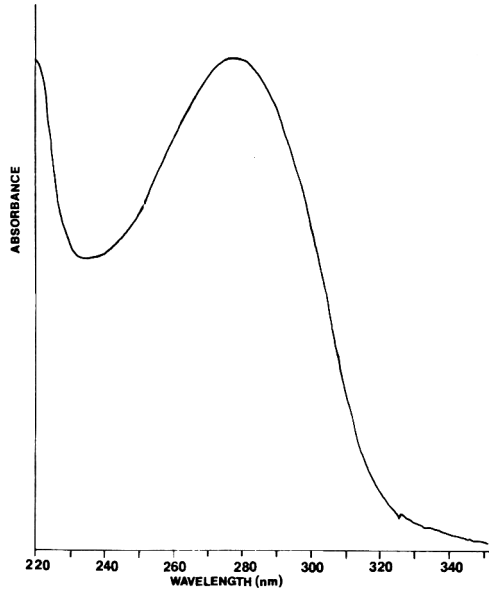


Figure 4. Ultraviolet absorption spectrum of *trans*-cinnamoylcocaine in mobile phase solvent system. Sample obtained by collection of peak 5 in Figure 1A.

trans-cinnamoylcocaine and the suspected *cis*-isomer are quite different. Moore (6) described the UV spectra for *cis*- and *trans*-cinnamoylcocaine as the same. However, this seems unlikely since in cases of *cis*-,*trans*-isomerism, the *trans*-isomer absorbs at the longer wavelength with the greater intensity (13). The UV spectra and the A254/A280 ratios obtained in this study do not support Moore's observation. The absorbance ratio obtained for the *trans*-isomer is much lower than that of the suspected *cis*-cinnamoylcocaine.

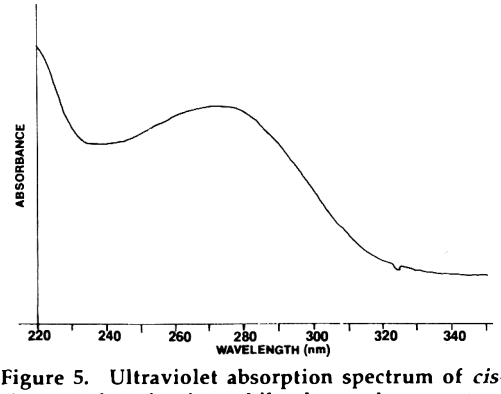


Figure 5. Ultraviolet absorption spectrum of *cis*-cinnamoylcocaine in mobile phase solvent system. Sample obtained by collection of peak 4 in Figure 1A.

The lower absorbance ratio would be expected for the *trans*-isomer which has the more intense absorption band at the higher wavelength (280 nm). These results are further supported by literature data (14) for the *cis*- and *trans*-isomers of cinnamic acid. The *cis*-isomer shows a wavelength of maximum absorbance at 268 nm and a molar absorptivity of 10 700 while the *trans*-isomer has a maximum absorbance at 272 nm and a molar absorptivity of 15 900.

The A254/A280 ratio can be a useful aid in the identification of liquid chromatographic peaks. Baker et al. (15) have used this ratio to determine the identity of drugs having similar elution characteristics in an HPLC system. However, the interlaboratory use of these ratios should be approached with caution. For example, the A254/A280 ratio for cocaine is reported as 0.86 (15) and was determined to be 1.95 in this study. The ratio for diazepam has been reported as 6.04 (15) and 4.62 (16). These variations are not surprising because molar absorptivity and the wavelength of maximum absorbance vary with solvent composition, pH, and other factors (17). Thus, these ratios should be determined in an individual chromatographic system using reference standards.

The identity of the liquid chromatographic peaks for cocaine and the cinnamoylcocaine isomers was confirmed by mass spectrometry. The individual peaks were collected from the chromatographic effluent and were isolated as the free base. Electron impact mass spectrometry of the isolated residues produced a molecular ion at m/e 303 for the cocaine peak and at m/e 329 for the 2 peaks assigned to *cis*- and *trans*-cinnamoylcocaine. The mass spectrum for the cocaine peak was essentially identical to previously reported spectra (9, 18). No significant differences were observed between the mass spectra for *cis*- and *trans*-cinnamoylcocaine. *cis*- and *trans*-Isomers usually have very similar mass spectra (18). The mass spectrum obtained for *trans*-cinnamoylcocaine is shown in Figure 6.

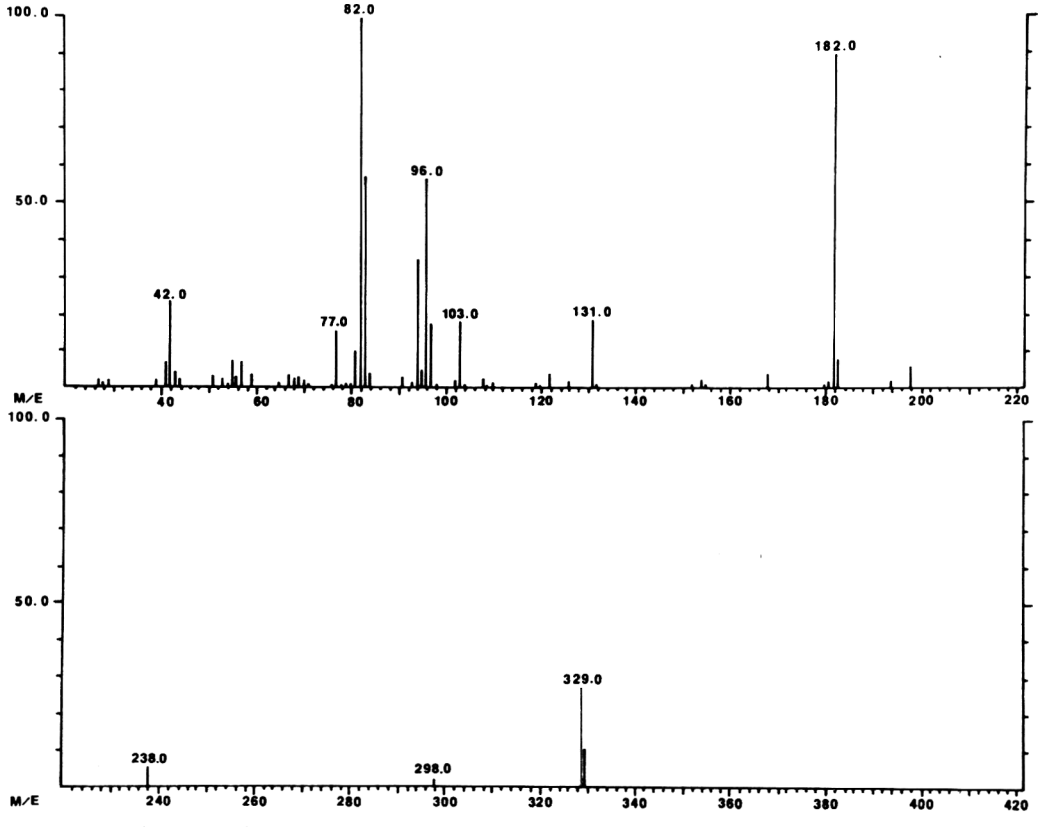


Figure 6. Electron impact mass spectrum of isolated *trans*-cinnamoylcocaine.

The lower mass ions observed in the fragmentation of the cinnamoylcocaines are essentially identical to those for cocaine because they arise from the tropane (ecgonine) portion of the molecule. The molecular ion and the ion at m/e 298 (M-31) contain the cinnamoyl groups and are therefore characteristic for the cinnamoylcocaine isomers. The m/e 298 ion results from the loss of the methoxy group from the molecular ion. The corresponding ion in cocaine occurs at m/e 272. The ion at m/e 238 (M-91) can be produced by the loss of a benzyl group from the cinnamoyl portion of the molecule. No M-91 ion was observed in the fragmentation of cocaine. The presence of the cinnamoyl ion at m/e 131 and the styryl ion at m/e 103 is also characteristic for the isomeric cinnamoylcocaines. In cocaine the corresponding benzoyl and phenyl ions occur at m/e 105 and 77, respectively. A low intensity peak at m/e 77 was observed in the spectra of the cinnamoylcocaines.

The described liquid chromatographic procedure can be used for the separation and identification of the individual components in illict cocaine samples including *cis*- and *trans*-cinnamoylcocaine. The use of A254/A280 ratios is an additional aid in the qualitative identification of the chromatographic peaks. The procedure produces good resolution between the various components of illicit cocaine. Derivatization procedures before analysis, as in gas chromatography, are not necessary, and liquid chromatography at ambient temperature should limit the problems of thermal decomposition of cocaine-type compounds. Furthermore, the nondestructive nature of UV detection allows the individual peaks to be collected for further analysis by ultraviolet spectrophotometry, thin layer chromatography, or mass spectrometry.

REFERENCES

(1) People v. Harper, CO, 562 P. 2nd 1112, 1977
(2) United States v. Wilburn, 549 F. 2d 734
(3) Lewin, A. H., Parker, S. R., & Carroll, F. I. (1980) *J. Chromatogr.* **193,** 371–380
(4) Allen, A. C., Cooper, D. A., Kiser, W. O., & Cottrell, R. F. (1981) *J. Forensic. Sci.* **26,** 12–26
(5) Wilson, C. O., Gisvold, O., & Doerge, R. F. (Eds) (1977) *Textbook of Organic Medicinal and Pharmaceutical Chemistry*, 7th Ed., J. B. Lippincott Co., Philadelphia, PA
(6) Moore, J. M. (1973) *J. Assoc. Off. Anal. Chem.* **56,** 1199–1205
(7) Moore, J. M. (1978) "The Application of Derivatization Techniques in Forensic Drug Analysis," Proceedings of the International Symposium on Instrumental Applications in Forensic Drug Chemistry, May 29–30, U.S. Government Printing Office, Washington, DC
(8) Moore, J. M. (1974) *J. Chromatogr.* **101,** 215–218
(9) Lukaszewski, T., & Jeffery, W. K. (1980) *J. Forensic Sci.* **25,** 499–507
(10) Jatlow, P., Van Dyke, C., Barash, P., & Byck, R. (1978) *J. Chromatogr.* **152,** 115–121
(11) Masoud, A. N., & Keupski, D. M. (1980) *J. Anal. Toxicol.* **4,** 305–310
(12) Lurie, I. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1035–1040
(13) Silverstein, R. M., & Bassler, G. C. (1967) *Spectrometric Identification of Organic Compounds*, 2nd Ed., John Wiley and Sons, New York, NY
(14) Dyer, J. R. (1965) *Application of Absorption Spectroscopy of Organic Compounds*, Prentice-Hall Inc., Englewood Cliffs, NJ
(15) Baker, J. K., Skelton, R. E., & Ma, C. Y. (1979) *J. Chromatogr.* **168,** 417–427
(16) Wittwer, J. D., Jr (1980) *J. Liq. Chromatogr.* **3,** 1713–1724
(17) Conners, K. A. (1975) *A Textbook of Pharmaceutical Analysis*, 2nd Ed., John Wiley and Sons, New York, NY
(18) McLafferty, F. W. (1973) *Interpretation of Mass Spectra*, 2nd Ed., W. A. Benjamin Inc., Reading, MA

TECHNICAL COMMUNICATIONS

Caution on Nitric Acid Digestion of Organic Samples in Closed Systems

OLE K. BORGGAARD and MARTA WILLEMS
Royal Veterinary and Agricultural University,
Chemistry Department,
Thorvaldsensvej 40,
DK-1871 Copenhagen V, Denmark

There is a risk of explosion when organic samples are digested with nitric acid in a closed vessel at elevated temperatures for elemental analysis. Sample size and the size, material, and thickness of bomb walls should be carefully considered. Digestion in an open flask may be a safer alternative.

There are several methods for decomposing organic samples for subsequent elemental analysis, including digestion with nitric acid at elevated temperatures. To reduce digestion time and to prevent loss of volatile elements, it has been recommended that digestion be carried out at 150°C (1, 2) or 130°C (3) in the stainless steel crucible-shaped cup lined with Teflon described by Bernas (4) for inorganic samples.

Because there is a risk of explosion, a note of caution against substantially changing recommended sample weight (1 g wet basis) or nitric acid volume (5 mL) is included in AOAC method **25.115-25.116** (1, 5). This method for an alternative digestion for mercury determination in seafood specifies 30–60 min digestion at 150°C. This digestion method has also been proposed for bakery products without specifying sample size (2), and for foods in general with a sample size of 0.3 g dry weight (6). For various plant materials, 1 g sample may be used, but for cellulose-rich samples only 0.4 g and 3–5 mL nitric acid should be used (7).

Despite the cautions and specifications, we still feel that some uncertainty exists. Furthermore, the consequence of using this digestion method with too large a sample may be a violent explosion. During a current investigation of methods for cobalt determination in feedingstuffs, the above-mentioned method seemed promising. Because of the limited cobalt content in grass and especially in grain, the solid:solution ratio must be high, increasing the risk of explosion. Therefore a special 25 mL digestion bomb was made in the laboratory, similar in construction to the Parr acid digestion bomb (8) but with a much heavier (10 mm) steel body, except the bottom flange which was 4 mm. A rough estimate showed that the bomb could resist a pressure of nearly 600 atm. before the bottom would blow out, compared with a maximum working pressure of aproximately 85 atm. for a Parr bomb (8). The bomb was first heated with 0.5 g dried grass and 5 mL nitric acid for 1 h at 150°C without damage. To improve the solid:solution ratio, 1 g dried grass and 5 mL nitric acid were put in the bomb, which was placed in a 150°C oven. After about 15 min an explosion occurred, perhaps due to the formation of nitrocellulose. The bottom of the bomb was forced through three 2 mm aluminum plates and through a 25 mm wood plate, before it was stopped against the laboratory floor.

This event emphasized that digestion with nitric acid in a closed vessel at an elevated temperature may be dangerous. The size, material, and wall thickness of the bomb as well as the type of organic material and the sample size must be judged very carefully before doing the experiment, e.g., as suggested by Parr Instrument Co. (8). Furthermore, it may be recommended to use bombs with a safety device so that the bottom blows out at over-pressure, and to carry out the digestion behind a protective shield. A safer method (9) specifies digestion in an open flask, using a combination of nitric and perchloric acids.

Received August 20, 1981. Accepted November 3, 1981.

REFERENCES

(1) Holak, W., Krinitz, B & Williams, J. C. (1972) *J. Assoc. Off. Anal. Chem.* **55**, 741–742
(2) Krinitz, B., & Holak, W. (1974) *J. Assoc. Off. Anal. Chem.* **57**, 568–569
(3) Holak, W. (1975) *J. Assoc. Off. Anal. Chem.* **58**,

0004/5756/82/6503/0762–02$01.00

777-780
(4) Bernas, B. (1968) *Anal. Chem.* **40**, 1682-1686
(5) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, **25.115-25.116**
(6) Holak, W. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 485-495
(7) Bock, R. (1979) *A Handbook of Decomposition Methods in Analytical Chemistry*, International Textbook Co., London, UK, 204 p.
(8) Parr Bulletin No 4745 (1976) Parr Instrument Co., Moline, IL
(9) Feldman, C. (1974) *Anal. Chem.* **46**, 1606-1609

Adsorption of 2,4-Dichlorophenoxyacetic Acid on Glassware as Source of Error in 2,4-D Analysis

GORDON Y. P. KAN, FRED T. S. MAH, and NORMAN L. WADE
Environment Canada, Inland Waters Directorate, Water Quality Branch, 1305 Welch St, North Vancouver, British Columbia, Canada V7P 1B3

Loss of 2,4-dichlorophenoxyacetic acid (2,4-D) by adsorption on Pyrex evaporating flasks and flint glass bottles was studied. 2,4-D may be quantitatively recovered by direct methylation with diazomethane in Pyrex evaporating flasks; it may also be desorbed with methylene chloride in the presence of strong H_2SO_4. This step must be taken when 2,4-D is transferred from one glass vessel to another to ensure accuracy of data.

The phenomenon of adsorption of phenoxy herbicides by organic and inorganic substrates is well documented (1-3). The adsorption of 2,4-dichlorophenoxyacetic acid (2,4-D), in particular, has been studied on a variety of adsorbents such as alumina, cellulose triacetate, cellulose powder, peat moss, silica gel, wheat straw, activated charcoal (4, 5), ion exchange resins (6), kaolinite, montmorillonite (7), and glass beads (8). Recently, while setting up a procedure to simultaneously analyze 2,4-D and 2,4-D butoxyethanol ether ester (2,4-D BEE), we experienced severe and unpredictable losses of 2,4-D in our precision and accuracy studies (9). We found that 2,4-D may be readily desorbed from glassware with methylene chloride in the presence of a strong mineral acid or by direct esterification of raw extract in its evaporating flask.

Experimental

Apparatus and Reagents

(a) *Gas chromatograph*—MT-220 equipped with linearized ^{63}Ni electron capture detector; 1.8 m × 4 mm id glass column packed with 3% OV-101 on 80-100 mesh Chromosorb W (Chromatographic Specialties Ltd, Brockville, Ontario). Operating conditions: injector temperature 190°C, column temperature 200°C, detector temperature 350°C, argon-methane (95 + 5) carrier gas 65 mL/min.

(b) *Diazomethane reagent.*—Prepared from Diazald, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Co.), in ethyl ether, using manufacturer's instruction and preparation kit.

(c) *Solvents.*—All solvents were pesticide grade (Burdick & Jackson Laboratories, Inc.).

(d) *Chemical standards.*—Herbicide standard compounds were obtained from the U.S. Environmental Protection Agency, Research Triangle Park, NC. Stock standards were prepared in acetone; working standards were prepared in isooctane.

Procedure

Recovery of 2,4-D from water.—A 2 L separatory funnel was charged with 1.0 L natural water (Capilano River, North Vancouver) which was spiked with 200 ng 2,4-D contained in 10 μL acetone. Sulfuric acid (10 mL, 50% v/v) was added and the water was vigorously extracted with two 200 mL portions of methylene chloride. The organic extract was collected in an Erlenmeyer flask, and then decanted into a 250 mL round-bottom flask for concentration. Isooctane (1-3 mL) was added and the extract was evaporated to ca 0.5 mL. Due to the large surface area of the evaporating flask, the extract was transferred to a 15 mL graduated centrifuge tube for esterification to avoid the use of a large excess of dia-

Received October 8, 1981. Accepted December 4, 1981.
Funding for this work was provided by the National Aquatic Weeds Program, National Water Research Institute, Burlington, Ontario.

0004-5756/82/6503-0763-03$01.00

Table 1. Recovery (%) of 2,4-D added to natural water[a]

Treatment	Replicates	Range	Mean	SD
1	3	45–80	62	18
2	3	85–97	91	6.0
3	3	93–98	97	3.2

[a] Before derivatization with diazomethane, raw extracts were treated in 3 different ways: Treatment 1—raw extract transferred from Pyrex evaporating flask to 15 mL graduated centrifuge tube for derivatization with diazomethane. Treatment 2—raw extract transferred in presence of H_2SO_4 from evaporating flask to graduated centrifuge tube for derivatization. Treatment 3—concentrated raw extract derivatized directly in evaporating flask.

zomethane, which would increase background interference during gas chromatographic/electron capture detection (GC/ECD) analysis. This was done by rinsing the round-bottom flask with three 2 mL portions of isooctane which were pooled into the centrifuge tube and concentrated to ca 0.2 mL with nitrogen. Diazomethane (5–10 drops) was added, and after 30 min at room temperature the excess diazomethane was purged with nitrogen. The residue was diluted to 10 mL with isooctane for GC analysis.

Raw extract storage study.—Because 2,4-D BEE was hydrolyzed rapidly to 2,4-D under neutral-to-basic conditions (10), we decided to pre-extract water samples in the field and ship the organic extracts to the laboratory for analysis. A storage study of 2,4-D and 2,4-D BEE was conducted by simulating field conditions in the following manner: Twenty-four flint glass bottles (500 mL capacity) were each filled with 200 mL methylene chloride that had been shaken vigorously with 1.0 L distilled water and 10 mL 50% H_2SO_4. This methylene chloride was then spiked with 200 ng 2,4-D and 400 ng 2,4-D BEE. The bottles were tightly stoppered and stored in the dark at 4°C. At 1 week intervals, 6 bottles were removed and the methylene chloride was emptied into six 250 mL round-bottom flasks to be concentrated and derivatized directly in the flask with diazomethane.

Results

Severe losses of 2,4-D were experienced when recoveries from water samples were carried out as described above. However, we found that quantitative recoveries of 2,4-D could be achieved by one of the following: (*1*) adding 1.0 mL 50% H_2SO_4 to the round-bottom flask after the extract has been concentrated to ca 0.5 mL and then extracting the concentrate with three 2 mL portions of isooctane which are carefully separated by means of disposable pipet and transferred to a centrifuge tube for direct derivatization, or (*2*) derivatizing the concentrated raw extract in the round-bottom evaporating flask with ca 0.5 mL diazomethane. These results are shown in Table 1.

In the study to determine the effect of storage time on recovery of 2,4-D and 2,4-D BEE from raw extracts, recoveries of 2,4-D were erratic (Table 2, a–d). Unfortunately, the bottles used in the study were washed before it was realized that adsorption on flint glass was a problem. The experiment was repeated by preparing 6 bottles of methylene chloride that was spiked with only 2,4-D. After 4 weeks, 3 spiked samples were analyzed by first introducing 5 mL 5% H_2SO_4 to each of the flint glass bottles. The contents were shaken; the methylene chloride was separated from the acid by decantation into a 250 mL round-bottom flask, and then concentrated and derivatized. No H_2SO_4 was added to the remaining bottles which served as controls. The results are shown in Table 2, e and f.

Discussion

Methylene chloride is a good solvent for extraction of 2,4-D and 2,4-D BEE from water under acidic conditions. Both of these phenoxy her-

Table 2. Recovery (%) of 2,4-D and 2,4-D BEE from raw extracts stored in flint glass bottles at 4°C

Treatment[a]	Time, weeks	No. of replicates	200 ng 2,4-D/L			400 ng 2,4-D BEE/L		
			Range	Mean	SD	Range	Mean	SD
(a)	1	6	30–98	78	26	91–102	98	4.3
(b)	2	6	40–119	80	33	93–109	99	5.6
(c)	3	6	62–109	91	24	101–106	103	2.1
(d)	4	6	29–108	84	28	96–107	102	3.9
(e)	4	3	14–91	49	39	unfortified		
(f)	4	3	95–110	101	8.0	unfortified		

[a] a–e: No H_2SO_4 added to bottles before transferring the methylene chloride to round-bottom flasks for evaporation; f: H_2SO_4 added to bottles to desorb 2,4-D.

bicides appear to be stable in this solvent over a period of 4 weeks at 4°C (Table 2).

During esterification of 2,4-D, it is important not to transfer the concentrated raw extract from the evaporating flask to a smaller container such as a 15 mL graduated centrifuge tube unless the transfer is preceded by desorption through deactivation of the walls of the flask with strong H_2SO_4. If this condition is met, the procedure becomes cumbersome in routine practice because then extreme care is needed to avoid transferring H_2SO_4 with the extract, which might destroy the diazomethane. Derivatization of 2,4-D may be satisfactorily performed in the evaporating flask by adding sufficient diazomethane to completely wet the walls of the flask.

When flint glass bottles are used in the field for storing 2,4-D extracts, these bottles must be deactivated with strong H_2SO_4 during sample workup in the laboratory. Caution must always be taken to prevent adsorption when transferring 2,4-D from one glass vessel to another. Such a transfer must be done in the presence of a strong mineral acid or after the 2,4-D has been esterified. Inclusion of this precaution in existing analytical procedures is the only way to ensure accuracy of data.

Acknowledgment

The authors thank W. Erlebach, Chief of Water Quality Branch, Pacific and Yukon Region, for reviewing this manuscript.

REFERENCES

(1) Haque, R., & Sexton, R. J. (1968) *Colloid Interface Sci.* **27**, 818–827
(2) Khan, S. U. (1972) *Environ. Lett.* **4**, 141–148
(3) Grover, R., & Smith, A. E. (1974) *Can. J. Soil Sci.* **54**, 179–186
(4) Coffey, D L., & Warren, G. E. (1969) *Weed Sci.* **17**, 16–19
(5) Weber, W. J., & Gould, J. P. (1966) *Adv. Chem. Ser.* **60**, 280–309
(6) Renberg, L. (1974) *Anal. Chem.* **46**, 459–461
(7) Bailey, G. M., White, J. L., & Rothberg, T. (1968) *Proc. Soil Sci. Soc. Am.*, **32**, 222–234
(8) Osadchuk, M., Salahub, E., & Robinson, P. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 1324–1327
(9) Kan, G. Y. P., Mah, T. S., Wade, N., & Bothwell, M. L. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 1305–1308
(10) Zepp, R. G., Wolf, N. L., Gordon, J. A., & Gaughman, G. L (1976) *Environ. Sci. Technol.* **10**, 1144

Evaluation of Tabarie's Formula

FABIÁN ALVAREZ-NAZARIO
Treasury Department of Puerto Rico, Bureau of Alcoholic Beverages Taxes, San Juan, Puerto Rico 00905

A strict derivation of an empirical formula, long used in the wine industry for the determination of extract, shows its inherent limitations.

Attempts to make a direct determination of the dissolved solid matter in wine by evaporation and drying (the so-called extract) will likely result in a viscous, sticky mass unsuitable for weighing. Two alternative methods have been traditionally used and accepted as AOAC official methods (1). In both methods, the specific gravity of the de-alcoholized wine, reconstituted to its original volume, is determined and the percent solids in the solution is found from standard tables of the specific gravity of sucrose solutions. Both methods take for granted that the usual solid matter dissolved in wine behaves like sucrose in its specific gravity effects.

In the first method, an actual determination is made of the specific gravity of the de-alcoholized wine (D). In the second method, D is determined from the empirical formula

$$D = S + 1 - S' \qquad (1)$$

where D = specific gravity of the de-alcoholized wine; S = specific gravity of the original wine; S' = specific gravity of a distillate of the wine.

This formula, known as Tabarie's formula, was proposed 150 years ago as an indirect method for determining alcohol in wine (2), but later was "turned around" by Fresenius and Grunhut (3) and used as an indirect method for determining the extract. Because routine analysis of wine requires the determination of specific gravity of

Received August 4, 1981. Accepted December 8, 1981.

0004-5756/82/6503-0765–03$01.00

both the original wine and of a distillate, the latter being a necessity for the alcohol determination, the specific gravity, D, and hence the amount of extract, is obtained as a bonus.

The purpose of this paper is to provide a derivation of Tabarie's formula, which will explain its shortcomings when it is applied to samples with high concentrations of alcohol and solids.

Derivation of the Formula

If we remove the extract from 100 mL of a solution of alcohol, water, and extract, and add water to bring the volume back to 100 mL, we obtain the relationship

$$100\,s - e + V_1 d_{H_2O} = 100\,s' \qquad (2)$$

where s = density (g/mL) of the original alcohol–water–extract solution; e = mass of extract/100 mL original solution; V_1 = volume of water needed to bring the volume back to 100 mL; d_{H_2O} = density of water; s' = density of the equivalent of 100 mL distillate.

If we remove that same extract from 100 mL of water–extract solution and add water to bring the volume back to 100 mL, we have

$$100\,d - e + V_2 d_{H_2O} = 100\,d_{H_2O} \qquad (3)$$

where d = density of the equivalent of 100 mL de-alcoholized solution; V_2 = volume of water needed to bring the volume back to 100 mL.

Subtracting equation (3) from equation (2), dividing by 100, and from the density of water and by rearranging, we have in terms of specific gravities

$$D = S + 1 - S' + (V_1 - V_2)/100 \qquad (4)$$

From equation (4) it is seen that Tabarie's formula will be true only if $V_1 = V_2$, i.e., if the amount of water needed to compensate for the volume loss which occurs on removing the extract from solution will be the same whether the extract is removed from an alcoholic solution or from a water solution. The term $(V_1 - V_2)/100$ will give an error when Tabarie's formula is used if such is not the case. An idea of the magnitude of this error can be obtained from published data on the specific gravity of alcohol–water–sucrose solutions. From the composition by weight and the density in g/mL of such solutions, we can readily obtain g alcohol/100 mL, i.e., the composition of a distillate, and g sucrose/100 mL, which is equivalent to the composition of the de-alcoholized solution. Existing alcoholimetric and saccharimetric tables (4) can be modified to give composition in terms of grams of alcohol or of sucrose/100 mL vs specific gravity.

Thus, from direct experimental data we obtain S and by suitable interpolation from existing tables we can obtain S' and D. The term $(V_1 - V_2)/100$ can then be obtained from equation (4).

Calculated values of $(V_1 - V_2)/100$ are given in Table 1. Data for high sucrose concentrations were obtained from the extensive work of Kharin

Table 1. Error in Tabarie's formula when applied to the system alcohol–water–sucrose [a]

Wt % alcohol	Wt % sucrose	$S_{20/20}$	$D_{20/20}$	$S'_{20/20}$	$(V_1 - V_2)/100$
19.08	5	0.98975	1.01908	0.97110	0.00043
18.07	10	1.01017	1.03884	0.97198	0.00065
17.07	15	1.03129	1.05935	0.97288	0.00094
16.06	20	1.05353	1.08065	0.97382	0.00094
14.06	30	1.10145	1.12587	0.97798	0.00240
36.00	20	1.01162	1.07747	0.93931	0.00516
33.75	25	1.03512	1.09886	0.94269	0.00643
31.50	30	1.05947	1.12114	0.94609	0.00776
29.25	35	1.08528	1.14441	0.94950	0.00863
27.00	40	1.11174	1.16861	0.95294	0.00981
24.75	45	1.14025	1.19410	0.95632	0.01017
33.179	0.107	0.95024	1.00039	0.94994	0.00009
33.144	0.213	0.95066	1.00079	0.94998	0.00011
33.073	0.426	0.95150	1.00157	0.95006	0.00013
32.916	0.898	0.95337	1.00333	0.95024	0.00020
32.716	1.680	0.95636	1.00622	0.95042	0.00028
32.178	3.293	0.96297	1.01224	0.95102	0.00029

[a] $S_{20/20}$ = specific gravity in vacuum of original alcohol–water–sucrose solution; $D_{20/20}$ = specific gravity in vacuum of de-alcoholized solution; $S'_{20/20}$ = specific gravity in vacuum of distillate.

Data for solutions containing 5–45 wt % sucrose from ref. 5; data for 0.107–3.293 wt % sucrose from experimental work in this laboratory.

and Tselinskaya (5). For very low sucrose concentrations, the experimental work was done in this laboratory by mixing known weights of pure sucrose with alcohol–water mixtures of known concentration and weighing the resultant mixture again on an analytical balance. Specific gravities of the mixtures were determined at 20°C using pycnometers. Percentage compositions and specific gravities were corrected to vacuum conditions.

It is evident from values in Table 1 that Tabarie's formula is only a convenient approximation, useful perhaps for dry wines, but which must be used with caution in other cases.

References

(1) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, sec. **11.014**
(2) Tabarie, E. (1830) *Pogg. Ann. Physik Chem.* **20,** 625
(3) Fresenius, W., & Grunhut, L. (1912) *Z. Anal. Chem.* **51,** 23–52
(4) "Standard Density and Volumetric Tables" (1924) Circular NO. 19, National Bureau of Standards, Washington, DC, Tables 1 and 12
(5) Kharin, S. E., & Tselinskaya, V. I. (1961) *Iz. Vyssh. Uchebn. Zavad. Pishch. Tekhnol.* **3,** 138–142

FOR YOUR INFORMATION

Journal Goes to Multiple Editor System

With regret, AOAC accepted Helen Reynold's resignation as Editor of the *Journal of the AOAC*, effective March 1, 1982. Ms Reynolds, head of the Food and Drug Administration's Technical Editing Group, has been associated with the *Journal* since 1953 and Editor since 1970. She is also immediate past president of AOAC. When Ms Reynolds left, the *Journal* also lost Norma Yess, also with the Technical Editing Group, who resigned her position as Associate Editor. They will both be sorely missed.

To fill the vacancy, AOAC decided on a multiple editor system. Five editors have been chosen. Each will be responsible for the peer review process and judgment of manuscript merit in a technical area in which the editor is actively involved.

Rodney J. Noel, the Agricultural Materials Editor, is Laboratory Director at the Office of Indiana State Chemist and Seed Commissioner, Purdue University, Department of Biochemistry, West Lafayette, IN 47907. He has been with the Indiana State Chemist Office since 1965. Active in AOAC, he has acted as a reviewer of AOAC publications and is presently Associate Referee for Protein in Animal Feeds, General Referee for Drugs in Feeds, and a member of the Committee on Safety. Outside AOAC, he is on the AAFCO Check Sample Committee, the AAPCO Methods Clearinghouse Committee, and the AVC Vitamin A Committee.

Evelyn Sarnoff, the Drugs, Colors, Cosmetics, and Forensic Sciences Editor, has been a Research Coordinator for the Food and Drug Administration, 850 Third Avenue, Brooklyn, NY 11232, for the past 9 years. Before that, she was Supervisory Chemist with a Food and Drug Administration pharmaceutical analysis laboratory. Ms Sarnoff has been an instructor in the Pharmaceutical Analysis College of Pharmaceutical Sciences, Columbia University, for 5 years. She is active in AOAC as Associate Referee, General Referee, Committee member, and Committee Chairman—all in the area of pharmaceutical analysis. In 1969, she was made a Fellow of the AOAC. Other organizations in which she participates include the American Chemical Society, the American Institute of Chemists, and the Eastern Analytical Symposium.

Malcolm C. Bowman, the Food Contaminants and Biological Methods Editor, recently retired from his position as Director of the Division of Chemistry, Food and Drug Administration, National Center for Toxicological Research, Jefferson, AR 72079. He presently heads Bowman and Associates, consulting scientists. Before joining FDA in 1962, he was associated with the Agricultural Research Service of the U.S. Department of Agriculture, the Campbell Soup Co., and the Army Chemical Corps. In addition to having almost 200 papers published, Malcolm Bowman has been on the Advisory Board of the *Journal of Agricultural and Food Chemistry*, the Editorial Board of the *International Journal of Environmental Analytical Chemistry*, Adjunct Professor of Chemistry at Murray State University. Murray, KY, and Adjunct Assistant Professor of Entomology at the University of Georgia.

Joseph Sherma, Residues and Elements Editor, is a professor of analytical chemistry at the Lafayette College Department of Chemistry, Easton, PA 18042. His continued research has been the basis for the more than 200 papers, books, and book chapters concerned with chromatography which he has authored or co-authored. He is editor of the Kontes TLC newsletter and is a consultant for several industrial companies and federal agencies on chemical analysis and chromatography, and regularly referees papers for analytical journals and research proposals for government agencies.

James F. Lawrence, the Food Composition and Additives Editor, is Head of the Food Additives and Contaminants Section, Health and Welfare Canada, Health Protection Branch, Food Research Division, Tunney's Pasture, Ottawa, Ontario K1A 012 Canada. Before that, he spent 8 years with the Pesticide Section working on pesticide residue methodology. He has been a Regional Editor of the *Journal of Environmental Analytical Chemistry* for the past 5 years, and author, co-author, or editor of a number of books, book chapters, and over 70 research papers on analytical methods. At present, his research emphasis is focused on analytical methodology

for fruit and vegetable coatings, food colors, emulsifiers, conditioning agents, brominated oils, and polycyclic aromatic hydrocarbons.

AOAC's List of Private Sustaining Members Continues to Grow

AOAC welcomes the latest additions to the growing list of companies supporting independent methods validation: Cargill, Inc., Minneapolis, MN; Hoffmann-La Roche, Inc., Nutley, NJ; McKee Baking Co., Collegedale, TN; Ralston Purina Co., St. Louis, MO; Salsbury Laboratories, Inc., Charles City, IA; and Schenley Distillers, Inc., Cincinnati, OH.

Alberta is First

The Alberta Department of Agriculture, Edmonton, Alberta, Canada, is the first provincial agency to provide AOAC with financial support. AOAC welcomes the Alberta Department of Agriculture as a provincial member of AOAC.

Magruder Fertilizer Check Sample Program Evaluating Laboratory Performance After 60 Years

Originated in 1922 by E. W. Magruder, the Magruder Fertilizer Check Sample Program is still providing laboratories with interlaboratory evaluation of methods of analysis and personnel performance.

Today a total of 140 U.S. industrial and foreign laboratories and all state control laboratories subscribe. Each month the subscribing laboratories receive one or two commercial fertilizer samples containing primary plant nutrients—nitrogen, potassium, and phosphorus—and, in addition, several times a year, secondary nutrients and micronutrients. Samples are analyzed by methods chosen by the individual laboratories. Results are analyzed by the Magruder statistician who prepares a statistical report for each laboratory, based on comparison of laboratories which used the same analytical method, noting interlaboratory bias, precision, and ranking of coded individual laboratories.

Interlaboratory evaluation is increasingly important as a check on instrument performance and to promote quality assurance. The program is strongly supported by the Association of American Plant Food Control Officials (AAPFC) and The Fertilizer Institute (TFI).

The annual subscription fee for this service is $75.00. For more information, contact Howard P. Moore, Feeds and Fertilizers, Department of Agriculture, Reynoldsburg, OH 43068.

ISO Standards Available from ANSI

The following is a list of standards from the International Organization for Standardization (ISO), Technical Committee 34-Agricultural Food Products. The Standards are available at prices indicated from American National Standards Institute, Inc., 1430 Broadway, New York, NY 10018.

ISO 663-1981	Animal and vegetable fats and oils—Determination of insoluble impurities and content	$ 6.30
ISO 751-1981	Fruit and vegetable products—Determination of water-insoluble solids content	7.80
ISO 1237-1981	Mustard seed—Specification	16.80
ISO 1736-1981	Dried milk—Determination of fat content (Reference method)	8.40
ISO 1737-1981	Evaporated milk and sweetened condensed milk—Determination of fat content (Reference method)	8.40
ISO 2166-1981	Carrots—Guide to storage	7.80
ISO 2167-1981	Round-headed cabbage—Guide to storage	9.75
ISO 2169-1981	Fruits and vegetables—Physical conditions in cold stores—Definitions and measurement	11.70
ISO 5223-1981	Test sieves for cereals	8.40
ISO 5492/4-1981	Sensory analysis—Vocabulary—Part 4	8.40
ISO 5521-1981	Fruits, vegetables, and derived products—Qualitative method for the	

	detection of sulfur dioxide	6.30
ISO 5522-1981	Fruits, vegetables, and derived products—Determination of total sulfur dioxide content	13.65
ISO 5523-1981	Liquid fruit and vegetable products—Determination of sulfur dioxide content (Routine method)	8.40
ISO 5561-1981	Caraway, whole—Specification	6.30
ISO 6000-1981	Round-headed cabbage—Storage in the open	10.50
ISO 6322/2-1981	Storage of cereals and pulses—Part 2: Essential requirements	10.50
ISO 6541-1981	Agricultural food products—Determination of crude fiber content—Modified Scharrer method	8.40
ISO 6579-1981	Microbiology—General guidance on method for the detection of *Salmonella*	21.00
ISO 6632-1981	Fruits, vegetables, and derived products—Determination of volatile acidity	15.60
ISO 6644-1981	Cereals and milled cereal products—Automatic sampling by mechanical means	10.50
ISO 6659-1981	Sweet pepper—Guide to refrigerated storage and transport	8.40
ISO 6821-1981	Greenhouse tomatoes—Guide to refrigerated transport	6.30
ISO 6882-1981	Asparagus—Guide to refrigerated transport	6.30

Meetings

May 25–27, 1982: Nitrogen in Crop Production Symposium, Holiday Inn, Sheffield, AL. Sponsored by TVA's National Fertilizer Development Center, American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America. Organized to provide a comprehensive summary of current knowledge about nitrogen as it relates to crop production. Emphasis will be placed on practical ways to improve plant use of nitrogen in different cropping systems. The proceedings, augmented by additional topics, will be published within 6 months after the symposium. Registrants will receive one copy, the cost of which is included in the $40 registration fee. For information, contact Helen Baugh, T-7B, National Fertilizer Development Center, Tennessee Valley Authority, Muscle Shoals, AL 35660.

May 25–27, 1982: World Symposium on Asbestos, Montreal, Quebec, Canada. Seventy-two speakers and panelists from government, unions, industry, and scientific and medical community from 14 different countries. For further information, contact the Secretariat, World Symposium on Asbestos, 84 de Brésoles St, Montreal, Quebec, Canada H2Y 1V5; telephone 514/845-6165; telex 05-24245.

June 2–3, 1982: Midwest Regional AOAC Meeting, Starlite Village Motel, Ames, IA. To include exhibit and technical sessions. For details, contact H. M. Stahr, 1636 College of Veterinary Medicine, Iowa State University, Ames, IA 50011; telephone 515/294-1950.

June 6–11, 1982: International Symposium on the Synthesis and Applications of Isotopically Labeled Compounds, Hyatt-Regency Hotel, Kansas City, MO. Topics will encompass synthesis, analysis, purification, and storage of isotopically labeled compounds, and their applications in biomedical, clinical, and environmental studies, in metabolism, pharmacokinetics, and toxicology. Proceedings of the symposium will be published ($35/copy). Registration fee: $220. For information, contact Alexander Susan, Scientific Secretary of the Symposium, c/o Midwest Research Institute, 425 Volker Blvd, Kansas City, MO 64110; telephone 816/753-7600, extension 268.

June 6–11, 1982: ASTM E-14 Meeting on Mass Spectrometry, Hilton Hawaiian, Honolulu, HI. For information, contact Louise Neall, 215/299-5400.

June 15–18, 1982: General Assembly of the Technical Committee of IOCC/ISCMA (International Office of Cocoa and Chocolate/ International Sugar Confectionery Manfacturers' Association), Gartenhotel Tummlerhof, Seefeld, Austria. Registration fee: 3.200 Austrian Shillings. For more information, contact Emile Toebosch, Director General IOCC/ISCMA, 172 avenue de Cortenbergh, B-1040 Brussels, Belgium.

June 16–18, 1982: 2nd International Symposium on Chromatography, Szeged, Hungary. Papers will be published by the Hungarian Academy of Sciences; official language is English. For more information, contact Halemm J. Issaq, Frederick Cancer Research Center, PO Box B, Frederick, MD 21701, or Tibor Devenyi, Institute of Biochemistry, Hungarian Academy of Sciences, Budapest XI, Hungary.

June 20–23, 1982: International Conference on Chromatography and Mass Spectrometry in Biomedical Sciences, Grand Hotel del Mare, Bordighera, Italy. For information, contact Alberto Frigerio, Gruppo Italiano di Spettrometria di Massa in Biochimica e Medicina, Via Eritrea, 62, 60157 Milano, Italy.

September 20–23, 1982: Symposium on Food Research and Data Analysis, Voksenasen Hotel, Oslo, Norway. Focus on the development and use of computer-aided analysis of multivariate food research data. Fee: US $360, NKr 1800. For information, contact Symposium Secretariate: Norwegian Food Research Institute, Bjorn Eldstuen, PO Box 50, N-1432 Aas-NLH, Norway.

September 23–24, 1982: 5th European Seminar on Quality Control in the Pharmaceutical and Cosmetic Industries—Administrative and Economic Problems, University of Geneva, Switzerland. Main topics: Quality Cost Systems, Introduction and Implementation of Electronic Data Processing into Quality Assurance, and the Use of Electronic Data Processing in Quality Assurance Operations. For further details, contact the Economics of Quality Control Pharma Cosmetic Section, c/o SAQ, PO Box 2613, CH-3001 Berne, Switzerland; Telex: 33528 atag ch; Tel.: 031 22 03 82.

October 12–14, 1982: Dioxin 82—3rd International Symposium/Workshop on Chlorinated Dioxins and Related Compounds, International Conference Centre, Salzburg, Austria. Topics: Incineration sources; analysis and standards; fate, distribution, and levels; laboratory safety and disposal practices; toxicology and risk assessment; legal and regulatory aspects. Proceedings will be published. For information, contact E. Merian, Im Kirsgarten 22, CH—4106 Therwil, Switzerland.

December 6–8, 1982: Third Bi-annual International Thin Layer Chromatography Symposium—Advances in Thin Layer Chromatography, Hilton Hotel, Parsippany, NJ. Papers accepted. For details, contact J. C. Touchstone, Hospital University of Pennsylvania, Philadelphia, PA 19104, telephone 215/662-2081; or H. M. Stahr, 1636 College of Veterinary Medicine, Iowa State University, Ames, IA 50011, telephone 515/ 294-1950.

June 7–10, 1983: 1st International Symposium on Drug Analysis—From Pharmaceutical Preparation to Drug Monitoring, Free University of Brussels, Brussels, Belgium. Organized by the Belgian Society of Pharmaceutical Sciences. Topic: naturally occurring drugs, analytical problems in drug formulation development, control of pharmaceutical specialties, determination of drugs in food products for human or animal use, determination of drugs in biological media including drug monitoring, and research into toxic reactions due to drugs. Languages: English, French, and Dutch. For additional information, contact C. Van Kerchove, Scciété Belge des Sciences Pharmaceutiques—Belgisch Genootschap voor Pharmaceutische Wetenschappen, rue Archimedesstraat 11, B-1040 Brussels, Belgium; telephone (02) 733 98 20 ext. 33.

July 17–23, 1983: SAC 83—International Conference and Exhibition on Analytical Chemistry, the University of Edinburgh, Scotland. Topics: atomic spectroscopy, biochemical methods, chromatography, electroanalysis, electrophoresis, enzyme techniques, immunoassay, mass spectrometry, microanalysis, molecular spectroscopy, photoacoustic spectrometry, probe methods, radiochemistry, sample preparation, thermal methods, x-ray methods, automation, data processing, process control, microcomputers, microprocessors, and quality control. For information, contact P. E. Hutchinson, Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London, W1V OBN, UK.

July 27–30, 1983: 3rd International Conference on the Instrumental Analysis of

Foods and Beverages—Recent Developments, Corfu Hilton, Corfu, Greece. For information, contact D. J. Mussinan, IFF R & D, 1515 Highway 36, Union Beach, NJ 07735; telephone 201/264-4500.

Courses Offered

The American Chemical Society (ACS) is offering the following 2 courses at the Donaldson Brown Continuing Education Center, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061. Fees include tuition, lecture, laboratory materials, and lunches. For information, call 703/552-8322.

Microprocessors and Minicomputers, Interfacing and Applications.—June 6–11 and September 19–24, 1982. This is a hands-on course designed for engineers, scientists, and managers. No previous training in digital electronics or computers is necessary. Advanced hardware experiments and intermediate level software challenges are available for those with previous experience. Course time is split between lecture and practice. The fee is $665.00 for ACS members, $735.00 for nonmembers.

Polymer Chemistry, Principles and Practices.—August 22–27, 1982. Lecture and laboratory work will cover polymer synthesis, molecular weight determination, characterization of rheological and viscoelastic behavior, polymer structure and morphology, and mechanical testing. Elastomers, plastics, and fibers will be discussed. The fee is $695 for ACS members, $765 for nonmembers.

Design and Analysis of Scientific Experiments—The Massachusetts Institute of Technology will offer this one-week elementary course July 12–17, 1982. Applications will be made to the physical, chemical, biological, medical, engineering, and industrial sciences, and to experimentation in psychology and economics. The course will be taught by Professors Harold Freeman and Paul Berger. For information, contact Director of Summer Session, Room E19-356, Massachusetts Institute of Technology, Cambridge, MA 02139.

Immunotoxicology.—This NATO-sponsored Advanced Study Institute will be held July 14–24, 1982, at Acadia University, Wolfville Nova Scotia, Canada. Lectures, workshops, etc. concerning the adverse effects of drugs and environmental chemicals on the immune system will be presented. Approaches to immunotoxicological testing and the importance of pharmacokinetics and biotransformation in immunotoxicology will also be considered. Financial assistance to cover travel expenses and accommodations will be available to selected applicants affiliated with universities and other nonprofit institutions from NATO countries. For details, contact P. W. Mullen, Director, NATO Immunotoxicology ASI, Kemic Bioresearch Laboratories Ltd, Unit 7, The Industrial Mall, PO Box 878, Kentville, Nova Scotia, Canada B4N 4H8.

Technology of Pesticide Analysis.—The University of California Extension, Davis Campus, will offer these hands-on laboratory sessions July 12–23, 1982. The fee is $1500. The course is designed for professionals working in the field of environmental toxicology, plant protection, pest management, and pesticide residue analysis. International participants may receive sponsorship through several agencies: USAID, World Bank, International Development Research Centre, Commonwealth Foundation, and Commonwealth Fund for Technical Cooperation. For further information, contact Adolf Braun, University Extension, University of California, Davis, CA 95616; telephone 916/752-6021.

Interim Methods Adopted

The following methods have been adopted interim official first action by the respective Committees and the Official Methods Board. The Association will vote on adoption of the methods as official first action at the 96th Annual Meeting, Oct. 25–28, 1982. New Method for Differentiating Members of the *Bacillus cereus* Group, by S. M. Harmon, Food and Drug Administration, Washington, DC; Determination of Chloride Concentration in Cheese, by L. J. Poortvliet, Boter- en Kaascontrolestation Friesland, Leeuwarden, The Netherlands, and W. Horwitz, Food and Drug Administration, Washington, DC; HPLC Determination of Minor Saccharides in Corn Sugar, by C. E. Engel et al., Corn Refiners' Association, Washington, DC; Correction Factor for Soluble Solids in Citrus Fruit Juices as Degrees Brix, by J. N. Yeatman et al. (*J. Assoc. Off. Anal. Chem.* 59, 368 (1976)) as submitted by F. E. Boland, Food and Drug Administration, Washington, DC.

Copies of the interim methods are available from the AOAC office.

BOOK REVIEWS

Laboratory Handbook of Chromatographic and Allied Methods. Edited by O. Mikes. From the Ellis Horwood Series in Analytical Chemistry, edited by R. A. Chalmoss and M. R. Masson. Published by Halsted Press, New York, NY, 1979. 769 pp. Price: $89.50. ISBN 0470-26399-7.

This is an updated version of a book written in Czechoslovakian and translated to English. The subject, chromatographic techniques, covers perhaps the most commonly used techniques in modern chemistry and biochemistry laboratories. It is essential for these laboratories to have a comprehensive detailed chromatography book on hand and I recommend the above title for this use.

The first chapter contains definitions of the various types of chromatography. Each of the other chapters describes one chromatographic technique in depth and includes detailed quantitative determinations, systems for different classes of compounds, and pertinent literature.

The chapter on adsorption chromatography describes adsorbents for gravity-flow and high pressure liquid chromatography (HPLC). This chapter also gives some aspects of HPLC, mostly in the form of examples.

The chapter on ion exchange chromatography discusses characteristics and theoretical principles of ion exchanges. It presents examples involving inorganic and small organic molecules, amino acids, antibiotics, and larger particles such as cell wall fractions. This rather complete review appears to cover the work through 1975.

In the chapter on gel chromatography, the examples given illustrate the basic principles, such as the dependence of molecular weight determination on the distribution constant of the solutes.

The chapter on affinity chromatography describes an important technique in modern biochemistry—the only solution for direct extraction of a biologically important molecule from a complex biological system. The technique is adequately described and the literature is reviewed up to 1974.

In 2 chapters, thin layer chromatographic equipment, adsorbents, solvent systems, and quantitative and preparative techniques are discussed. An inclusive chapter on gas chromatography mentions the possible combination of gas chromatography with spectral methods like mass spectrometry. A tabulation at the end of the chapter gives details and references of areas of application of gas chromatography.

The last chapter details electromigration methods. It discusses electrophoresis, isotachophoresis, and isoelectric focusing, mostly from the technical point of view and with many good examples.

This book is comprehensive and can serve as an excellent aid in laboratory investigations.

ADORJAN ASZALOS
Food and Drug Administration
National Center for Antibiotic Analysis
Washington, DC 20204

Treatise on Analytical Chemistry, Part II, Vol. 10, Analytical Chemistry of Inorganic and Organic Compounds. Edited by I. M. Kolthoff and P. J. Elving. Published by Wiley Interscience Publications, John Wiley and Sons, New York, NY, 1978. 566 pp. Price: $34.50. ISBN 0-471-49998-6.

This book is a compilation of laboratory methods and reference sources for the scientific analysis of antimony, arsenic, boron, carbon, molybdenum and tungsten. It is intended primarily for laboratory personnel, from the bench chemist to the university professor.

The methods are clearly and concisely written, and the principles of instrumentation are presented in terminology that can be comprehended by all scientists. The writers have compiled a selective and authoritative source of information.

The book is presented in a logical and well organized manner. There are 6 chapters. Each is dedicated to a specific element and provides pertinent history, occurrence, properties, and current legal limits. The writers are adept at presenting a consensus of opinion concerning toxicity of an element in spite of inconsistent findings and disagreement among workers. Also, the writers successfully condense laboratory procedures at the end of each chapter for the convenience of the analytical chemist. Extensive references are given at the end of each chapter.

An overall introduction to the book should be added in future editions. The authors

could explain why they group these 6 elements together. For the regulatory fertilizer analyst, it would have been preferable to combine 4 other essential nutrients with the boron and molybdenum.

In general, the positive aspects of this book greatly outweigh the negative. It is a valuable reference for those engaged in the analysis of the described elements.

JAMES R. MELTON

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

Reagent Chemicals. American Chemical Society Specifications. 6th Ed. Prepared by the Committee on Analytical Reagents (1975–1980) Samuel M. Tuthill, Chairman. Published by the American Chemical Society, Washington, DC, 1981. 612 pp. Price: $60.00. ISBN 0-8412-0560-4.

The history of this publication was described in a review of an earlier edition (*J. Assoc. Off. Anal. Chem.* (1956) **39**, 1018). Twelve new items have been added to this edition, including 3 solvents suitable for pesticide residue analysis. Hydrated salts are now covered as specific items. Many tests have been consolidated into the general test method section in the front of the book. Postage-paid cards are included for obtaining future supplements.

Because reagents used in *Official Methods of Analysis of the AOAC* must conform to the specifications in this volume, all users of the AOAC book of methods need to have access to *Reagent Chemicals*.

WILLIAM HORWITZ

Food and Drug Administration
Bureau of Foods
Washington, DC 20204

FDA Technical Bulletin No. 1: Principles of Food Analysis for Filth, Decomposition, and Foreign Matter. Edited by J. Richard Gorham. Published by the Food and Drug Administration, 200 C St SW, Washington, DC 20204, 1981. viii + 286 pp. Price: $7.50. GPO Stock Number 017-012-00298-6.

An updated replacement of Technical Bulletin No. 1: *Microscopic-Analytical Methods in Food and Drug Control* published in 1960, this is a concise, well organized reference book. It contains sections which cover concepts of food sanitation; descriptive and illustrative information on common food contaminants such as fungi, mites, insects, and hairs; general theory behind analytical methods used to recover such contaminants; and various microscopic techniques used to identify food contaminants. Hundreds of drawings, charts, and photographs are included.

Major changes from the 1960 publication include chapters on scanning electron microscopy, tests for metabolic wastes, and mites, and updates in analytical mycology and photographic techniques. A useful index is also provided. Excluded are drug-related topics and some of the detail in areas such as filth detection techniques and analytical entomology.

Although this book is written primarily for regulatory analysts and sanitarians, it can also provide valuable technical information to students in a food science curriculum and to analysts and quality assurance supervisors in the food industry. It is competently written and edited, ranking alongside its excellent predecessor. The purchase and use of this book by the above mentioned groups is highly recommended.

DONALD A. MASTROROCCO, JR

Hershey Chocolate Co.
19 East Chocolate Ave
Hershey, PA 17033

The Analysis and Control of Less-Desirable Flavors in Foods and Beverages. Proceedings of a 1980 American Chemical Society, Agricultural and Food Chemistry Div. symposium held at the Second Chemical Congress of the North American Continent. Edited by G. Charalambous. Published by Academic Press Inc., 111 5th Ave, New York, NY 10003. 358 pp. Price: $24.50. ISBN 0-12-169065-2.

According to the introduction to this book, "Flavor is a most sensitive index of the quality of our food, often influencing its total value in a definite manner."

The authors report on the detection and identification of flavor precursors and off-flavors in fish, cheese, soy protein, corn syrup, water, citrus juices, milk (including human), beer, and wine. Improved methods of volatile component separations are outlined and extensive bibliographies accompany each chapter.

Included are papers on meat flavor characteristics caused by feed, and handling and storage of beef, lamb, and pork; important

flavor components and accelerated flavor development in cheese; flavor contributions of corn syrup and soy proteins to processed foods; the influence of protein interaction on flavor; the control of bitter flavors; and the use of acidic oligo peptides. Public water supply, citrus juices, beer, and wine each has its own chapter.

Milk receives a great deal of attention. Analysis, control, and identification of flavors and off-flavors, and the effect of heat processing of milk on volatile compounds are covered in detail. The results of an initial survey on the off-flavors of human milk are given along with interesting suggestions on the possibility of imprinting and later-life flavor-conditioning.

The study of flavor-caused rejection of some foods in their natural state and the off-flavors developed through processing and storage is of particular interest to food technologists and chemists.

This book can provide members of regulatory bodies with a better understanding of the mechanisms and processes involved in the development of off-flavors. It is recommended reading and study for anyone concerned with links in the food flavor chain.

R. ARSENAULT

Florasynth Canada, Inc.
St. Laurent, Quebec, Canada

Pesticide Residues – A Contribution to Their Interpretation, Relevance and Legislation. By H. Frehse and H. Geissbuhler. Published by Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, NY 10523, USA, 1979. 100 pp. Price: $40.00. ISBN 0-08-023931-5.

Two symposia, "The Reliability of Residue Data," "The Interpretation of Residues and Residue Data as Related to Toxicology and Legislation of Pesticides" with 8 papers apiece were presented at the Pesticide Residues Section of the IVth International Congress of Pesticide Chemistry (IUPAC) and published as this book.

The first symposium deals with the accuracy and reproducibility of residue data, discussing such topics as field sampling, field spray efficiency, collaborative studies, quality assurance, and quantitative variation in results.

The second symposium covers acceptable daily intake of pesticide residues, toxicology, residue types, pesticide tolerances, and food acceptability.

This book provides the enforcement point of view on obtaining, treating, and interpreting residue data. Any regulatory agency, private laboratory, manufacturer, or legislator dealing with pesticide residues could benefit from this book.

JERRY E. FROBERG

Food and Drug Administration
1521 W Pico Blvd
Los Angeles, CA 90015

The Analysis of Explosives. By Jehuda Yinon and Samuel Zitrin. Published by Pergamon Press, Maxwell House, Fairview Park, Elmsford, NY 10523, 1981. 310 pp. Price: Hardcover $60.00, ISBN 0-08-023846-7; Flexicover $22.50, ISBN 0-08-023845-9.

The format of the book, written by leading authors in the field, is that of a review article. Each chapter represents a specific analytical technique of explosives. It is a book which is long overdue. Twelve of the 15 chapters are specifically concerned with individual analytical techniques. The last 2 chapters deal with explosive residues and hidden explosives.

The book consists of current and valuable scientific data and currently used methods for analysis of explosives. The discussion of chemistry and scientific theory applicable to the described methods is very informative. The book is presented in a well organized manner. It serves its intended purpose—as a reference book for chemists in analytical and forensic laboratories, as well as a textbook for graduate students in analytical chemistry and forensic science.

The book is printed from high quality original typescripts. The flexicover version is fairly priced at $22.50; but the hardcover is over- priced at $60.00.

JEW-MING CHAO

Burlington County Forensic Science Laboratory
Woodlane Rd
Mt. Holly, NJ 08060

The Pesticide Chemist and Modern Toxicology. A 1980 American Chemical Society, Division of Pesticide Chemistry symposium held at a special conference at Downington, PA. Edited by S. K. Bandal, G.

J. Marco, L. Golberg, and M. L. Leng. Published by the American Chemical Society, 1155 16th St NW, Washington, DC 20036, 1981. 582 pp. Price: $38.00. ISBN 0-8412-0636-8.

The conference at which this symposium was given was convened to provide a means for the disciplines of toxicology and pesticide chemistry to interact in a direct and personal way. A very convincing case for interdisciplinary interaction is presented in this volume.

Contents of the book (36 presentations) are divided into 4 major sections followed by a short summary of the discussion groups and workshops which took place. The sections are: Toxicological Aspects, Biochemical Aspects, Analytical Aspects, and Regulatory Aspects. The papers in each section present an excellent review of the discipline or subject, emphasizing the interdependence of chemistry and toxicology and the interdisciplinary nature of any meaningful research.

The well written papers provide a great deal of useful information, usually beginning with simple basic principles and progressing to detailed descriptions or reviews of the most recent research and research in progress. Extensive literature references are included. This book gives a very broad and encompassing view of pesticides, pesticide toxicology, and pesticide chemistry. It should be of interest to anyone involved in such research, and should be an invaluable asset to anyone involved in regulating pesticides in an analytical laboratory or a registration capacity.

KENNETH HELRICH

Rutgers University
Cook College
Dept of Entomology & Economic Zoology
New Brunswick, NJ 08903

NEW PUBLICATIONS

Calculator Programming for Chemistry and the Life Sciences. By Frank H. Clarke. Published by Academic Press, 111 5th Ave, New York, NY 10003, 1981. 240 pp. Price: $24.50. ISBN 0-12-175320-4.

This work illustrates the utility of the programmable calculator as a tool for scientific research. Specific, detailed examples from select topics in chemistry and biology demonstrate the interrelationship between these scientific disciplines. The use of atomic coordinates as measures of the degree of correspondence between 2 molecules of different molecular structure and a method for determining partition coefficients are described. Regression analysis is used to correlate the biological and physical properties of compounds. Programs are described for use in the laboratory. Although designed for the Texas Instrument T1-59 calculator, the book also provides several programs in Reverse Polish Notation for Hewlett Packard calculators.

Analytical Profiles of Drug Substances, Volume 10. Edited by Klaus Florey. Published by Academic Press, 111 5th Ave, New York, NY 10003, 1981. 752 pp. Price: $41.00. ISBN 0-12-260810-0.

The Pharmaceutical Analysis and Control Section of the Academy of Pharmaceutical Sciences has begun a cooperative venture to compile and publish analytical profiles of important drug substances. These profiles present information not currently available in official compendia, including the physical and chemical properties of drug substances, methods of synthesis and pathways of physical and biological degradation and metabolism, solubility, pH and pK values, spectra and spectrophotometric constants, and stability data. The profiles will be revised and updated when necessary.

Trace Analysis, Volume 1. Edited by James F. Lawrence. Published by Academic Press, 111 5th Ave, New York, NY 10003, 1981. 344 pp. Price: $39.50. ISBN 0-12-682101-1.

Trace Analysis is a multivolumed publication intended to bring together detailed applications of analytical chemistry to the detection, identification, and quantitation of trace quantities of substances in many different sample types. The serial includes articles that discuss new analytical instrumentation and techniques and approaches to methodology for solving specific trace analytical problems. Volume 1 is devoted to state-of-the-art critical discussions of selected topics in the use of high performance liquid chromatography for both organic and inorganic trace analysis.

Thin-Layer Chromatography Techniques and Applications. By B. Fried and J. Sherma. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1982. 328 pp. Price: $49.50. ISBN 0-8247-1288-9.

The authors present the principles, practices, and applications of thin layer chromatography (TLC). Designed for those without extensive training in analytical chemistry, this volume presents a self-teaching treatment of this technique, as well as a source of information for analytical chemists, biochemists, clinical chemists, and laboratory and medical technicians. It is also of use to zoologists and botanists interested in separation sciences and to college teachers of biology and chemistry.

Aquatic Toxicology and Hazard Assessment. Edited by D. R. Branson and K. L. Dickson. Published by American Society for Testing and Materials, 1916 Race St, Philadelphia, PA 19103, 1981. 466 pp. Price: $43.00. ASTM/STP 737. PCN: 04-737000-16.

The 31 papers contained in this manual are divided into sections on safety margins, hazard assessment, carcinogenesis in aquatic organisms, monitoring, statistical analysis of toxicity data, and new concepts. The book explores the need to develop relevant testing methodology and integrated testing programs to accurately assess the potential hazard of chemicals to aquatic life. This publication stresses that the term "hazard" is a function of both toxicity and exposure which makes it the essential linkage of aquatic toxicology with environmental chemistry.

Pesticide Residues in Food – 1980. Published by the Food and Agriculture Organization (FAO), 1981. Available from Unipub, 345 Park Ave S, New York, NY 10010. 79 pp.

Price: $7.50. FAO Plant Production and Protection Paper No. 26. Unipub Catalog Code No. 0102-F2180.

The recommendations of a joint meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues, Rome, Italy, October 1980, are outlined in this report. The recommendations include acceptable daily intakes (ADIs), maximum residue limits, and indications for further research and information.

Enzymes and Food Processing. A 1980 National College of Food Technology, University of Reading symposium. Edited by G. G. Birch, N. Blakebrough, and K. J. Parker. Published by Applied Science Publishers, Barking, Essex, UK, 1981. Distributed in the U.S. by International Ideas, Inc., 1627 Spruce St, Philadelphia, PA 19103. xii + 295 pp. 109 illus. Price: $44.00. ISBN 0-85334-935.

The papers from this symposium are concerned with the use of enzymes, indigenous and foreign, in processing both isolated substrates and complex products such as meat, milk, fruit juices, and bread. Health and safety aspects, detoxifying enzymes, and analytical applications are also discussed.

Dispersion of Powders in Liquids, 3rd Ed. Edited by G. D. Parfitt. Published by Applied Science Publishers, Barking, Essex, UK, 1981. Distributed in the U.S. by International Ideas, Inc., 1627 Spruce St, Philadelphia, PA 19103. xv + 510 pp. 109 illus. Price: $76.00. ISBN 0-85334-990-8.

In this, the 3rd edition, there has been a significant updating of the information provided by earlier editions. The book bridges the gap between the academic approach to the problems of dispersion and that which is used in industrial practice. It outlines the principles, discusses the technology, and assesses the current problems associated with the dispersion of powders in liquids, with particular reference to pigments. In addition, the book covers the fundamental aspects of the breakdown of solid materials, both amorphous and crystalline, and identifies the parameters that are important in comminution.

Natural Colours for Food and Other Uses. Proceedings of a 1979 Roche Products Ltd conference, London. Edited by J. N. Counsell. Published by Applied Science Publishers, Barking, Essex, UK, 1981. Distributed in the U.S. by International Ideas, Inc., 1627 Spruce St, Philadelphia, PA 19103. x + 173 pp. 26 illus. Price: $26.00. ISBN 0-85334-933-9.

Dealing with the highly sensitive subject of color in foods, this book should be of interest to color chemists, food technologists, food manufacturers, and cosmetic chemists. Several aspects of color discussed are the physicist's viewpoint, color's importance in food psychology, its role in cosmetics and in hospital pharmacy, carotenoids and their applications, and legislative considerations.

Developments in Food Preservation – 1. Edited by Stuart Thorne. Published by Applied Science Publishers, Barking, Essex, UK, 1981. Distributed in the U.S. by International Ideas, Inc., 1627 Spruce St, Philadelphia, PA 19103. xii + 271 pp. 99 illus. Price: $60.00. ISBN 0-85334-979-7.

An up-to-date review of the current state of food preservation is provided by this book. In addition to the unit operations of food preservation, this volume includes chapters on relevant aspects of packaging, on the selection of technology appropriate to developing countries, and on the storage and transport of fresh produce.

Fats and Oils: Chemistry and Technology. From the Symposium on Advances in Chemistry and Technology of Fats and Oils, 1979. Edited by R. J. Hamilton and A. Bhati. Published by Applied Science Publishers, Barking, Essex, UK, 1981. Distributed by International Ideas, Inc., 1627 Spruce St, Philadelphia, PA 19103. xii + 263 pp. 53 illus. Price: $48.00. ISBN 0-85334-915-0.

The contents of this book deal with studies of oxygenated acids, synthetic glycerides, prostaglandins, extraction and processing of oils and fats. In addition, there are chapters on application of fats in confectionery and new ways of modifying ruminant fats.

Wilson and Wilson's Comprehensive Analytical Chemistry. Edited by G. Svehla. **Volume XIII – Analysis of Complex Hydrocarbon Mixtures. Part A, Separation Methods; Part B, Group Analysis and Detailed Analysis.** By S. Hála, M. Kuraš, and M. Popl. Published by Elsevier

Scientific Publishing Co., PO Box 211, 1000 AE Amsterdam, The Netherlands, 1981. Distributed in USA and Canada by Elsevier North-Holland, Inc., 52 Vanderbilt Ave, New York, NY 10017. Part A about 382 pp. Part B about 400 pp. Price per part: US $95.75/Dfl. 225.00, Subscription Price per part: US $85.00/Dfl. 200.00. Part A ISBN 0-444-99736-9. Part B ISBN 0-444-99735-0.

Part A describes the following methods of separation of hydrocarbons used in the initial steps of the analytical procedures: distillation techniques, preparative variants of gas and liquid chromatography, methods with shape selectivity, and proven chemical separation methods. Part B covers spectroscopic, chromatographic, and chemical techniques used for group and detailed analysis of hydrocarbon materials. Analyses of important industrial hydrocarbons are summarized in a special review.

Part 31 Water, 1981 Annual Book of ASTM Standards. Published by the American Society for Testing and Materials, 1916 Race St, Philadelphia, PA 19103, 1981. 1516 pp. Soft Cover. Price: $53.00. PCN 01-031031-16.

Part 31 contains 182 standards for water. Many of the standards are listed in the EPA "Guidelines Establishing Test Procedures for the Analysis of Pollutants" under section 304 (h) of the Clean Water Act. The book discusses definitions, specifications, reagents, reporting results, sampling and flow measurement, general properties of water, inorganic and organic constituents, radioactivity, saline and brackish waters, sea waters, brines, microbiological examination, water-formed deposits, and water-treatment materials.

CORRECTION

J. Assoc. Off. Anal. Chem. (1981) **64**, 1305–1308, "Determination of 2,4-D Butoxyethanol Ether Ester and Its Degradation Products 2,4-Dichlorophenoxyacetic Acid and 2,4-Dichlorophenol in Sediment," by G. Y. P. Kan et al., p. 1306, line 9 under ***Sample Collection*** should read "Ten μL of an intermediate standard (ppm range)"

OBITUARY

Alfred D. Campbell

On February 22, Alfred (Al) D. Campbell, of the Food and Drug Administration, at the age of 62 suffered a fatal heart attack. He had been on assignment in Saudi Arabia to assess a U.S. supported venture to develop a food protection system there. Al's passing leaves a void at the FDA Bureau of Foods in experience and expertise that will be difficult to fill.

Al joined the Food and Drug Administration in 1962 during a period of ferment in that agency and was assigned to the then Division of Foods in the Bureau of Physical Sciences. One of his first tasks was to explore the potential for trouble arising from the recently isolated toxic mold byproducts called aflatoxins. Through Al's foresight and organizational ability, the FDA, and particularly the inter-disciplinary group that Al brought together and in one way or another managed to direct, became the leaders and acknowledged world-wide authorities in aflatoxin research and control. Their interests spread to other potential mold toxin problems, and eventually to all naturally occurring toxins in foods, as they pioneered work at FDA.

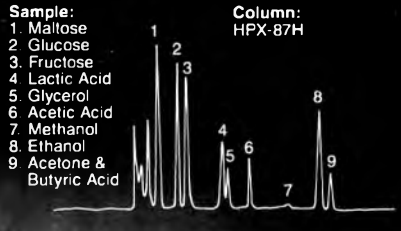
Al appreciated the value of organized method development and validation by an authoritative group, and early in his efforts involved the AOAC. In 1963, AOAC named Dr. Campbell Associate Referee for Aflatoxins under the General Referee for Decomposition and Filth in Foods. By 1966, Mycotoxins was established as a separate subject area with Al as General Referee. He held the position until 1969 when he moved to Subcommittee C, which had authority for mycotoxins topics. He was Committee C's mycotoxins authority at the time of his death.

Al made many friends who will now miss his help and advice. Along with his wife, two children, brother and sister, we mourn his passing.

LEONARD STOLOFF

Organic acid analysis can be difficult...

Or it can be simple with Bio-Rad HPLC.



Bio-Rad has worked out all the methodology for simple, rapid organic acid analysis and packaged it in one compact, affordable HPLC system. Here are some of the outstanding advantages of organic acid analysis with Bio-Rad HPLC:

- Versatile. Food, beverages, cosmetics, biomedical, process control.
- Sensitive and repeatable. To ppm level (more with preconcentration). Time and time again!
- Full technical support. Our staff of experienced separations chemists will help you optimize the application of your system and columns.
- Proven performance. Hundreds of Bio-Rad columns for organic acid analysis have delivered excellent results during the past two years.
- Affordable. Modular system gives flexibility of component selection, ease of set-up and service.

To us this seems like the simplest and most dependable method for organic acid analysis. That's understandable, since we've put a lot of thought into it. So you don't have to.

Call or write for details.

BIO·RAD *Laboratories*

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

In This Issue