Journal

of the

Association of Official Analytical Chemists

VOL. 59 NOVEMBER 1976 No. 6

CONTENTS

THE ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, INC.
Editorial and Advertising Offices: Box 540, Benjamin Franklin Station, Washington, DC 20044
Phone: Area Code 202/245-1526
Printed: 414 Water Street, Baltimore, MD 21202
Published: 200 C Street, S.W., Washington, DC 20204

OFFICERS

President: William W. Wright, Food and Drug Administration, Washington, DC 20204

Vice-President: Anthony J. Malanoski, U.S. Department of Agriculture, Washington, DC 20250

Treasurer: Bernhard Larsen, U.S. Department of Agriculture, Washington, DC 20250

Executive Committee: Elwyn Schall (Purdue University, W. Lafayette, IN 47907); W. Perce McKinley (Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario, Canada K1A 0L2); Helen Reynolds (Food and Drug Administration, Washington, DC 20204); and Ernest A. Epps, Jr. (Louisiana State University, Baton Rouge, LA 70803)

Executive Office

Executive Director: William Horwitz, Food and Drug Administration, Washington, DC 20204 (202/245-1301)

Executive Secretary: Luther G. Ensminger, Food and Drug Administration, Washington, DC 20204 (202/245-1191)

Scientist-Administrator: Douglas L. Park, Food and Drug Administration, Washington, DC 20204 (202/245-1266)

Methods Editor: Alan Senzel, AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20044 (202/245-1266)

Editorial Office (202/245-1526)

Editor: Helen L. Reynolds, Food and Drug Administration, Washington, DC 20204

Managing Editor: Nancy Palmer, AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20044

Associate Editor: Laura A. Kovách, Food and Drug Administration, Washington, DC 20204

Associate Editor: Norma Yess, Food and Drug Administration, Washington, DC 20204

Business Office (202/245-1266)

Business Manager: Rosalind H. Pierce, AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20044

Assistant Business Manager: Marjorie D. Fuller

Assistant Manager, Publications: Catherine A. Foster

INFORMATION FOR SUBSCRIBERS AND CONTRIBUTORS

The *Journal of the Association of Official Analytical Chemists* publishes research articles and reports of development and validation of analytical methods for foods, drugs, beverages, colors, cosmetics, agricultural products related to public health. *The Journal* is issued six times a year in January, March, May, July, September, and November, the six numbers comprising the annual volume. Each volume will contain approximately 1500 pages. Subscriptions are taken by the volume, at $36.00 prepaid in North America and U.S. possessions, and $40.00 to all other countries. Single copies of current issues are $8.00 plus $0.50 postage and handling.

Manuscripts should be typewritten, double-spaced, carefully revised before submission, and the original and two carbons submitted to the AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20044. "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 cost, 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, Box 540, Benjamin Franklin Station, Washington, DC 20044. 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., Alexander Rd, Princeton, NJ 08540; *see* p. 558 for more information.

METHODS VALIDATION

Problems of Sampling and Analytical Methods

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

The need for acceptable, reliable, and practical methods of analysis, chemical, physical, and biological, by the Food Standards Program of the Food and Agriculture Organization/World Health Organization is leading to cooperation by those organizations with the resources and experience to supply them. Methods should be shown to be workable and practicable and then validated in a properly designed international collaborative study for the efficient utilization of the time and effort of participating organizations and laboratories. The methods to be subjected to interlaboratory collaborative study should be clearly written so that the method itself is being tested without unauthorized variations. Satisfactory reference standard materials are often an essential part of the method. Uniform, international methods which have been developed through interlaboratory collaborative studies are applicable to the areas of microbiology and toxicology, as well as chemistry.

One of the least heralded achievements of the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Food Standards Program has been to provide an incentive to unify methods of analysis for food on an international basis. Most of the 14 commodity-oriented committees of the 23 subsidiary bodies of the Codex Alimentarius Commission have been sending recommended methods of analysis to the Codex Committee on Methods of Analysis and Sampling for endorsement. Approved methods are submitted through appropriate channels to governments for approval.

Over 15 years of experience has been attained in attempting to unify methods of analysis through this mechanism. The procedure began even before the establishment of the Codex Alimentarius Commission. The Joint FAO/WHO Committee of Government Experts on the Code of Principles Concerning Milk and Milk Products, which is now looked upon as the original commodity committee of the Codex, found themselves bogged down on the question of methods from their earliest sessions, which began in 1958.

The early meetings of the Committee of Experts displayed an intense competition from 3 primary organizations which were attempting to dominate the methods approval process. These organizations were:

The *International Dairy Federation* (*IDF*), the major international dairy organization, representing every country in the world with an important dairy industry, except the United States. This group had already promulgated a set of reference methods of analysis for use by their members.

The *International Organization for Standardization* (*ISO*), an international organization which was attempting to establish itself as the unifying nongovernmental group for standardization of all industrial products. Although their Agricultural Food Products Technical Committee (designated as TC 34) contained a Subcommittee on Milk and Milk Products (designated as SC 5), very few methods had made their way through the elaborate approval mechanism of ISO at that time. Because of regulatory constraints, the United States could not participate effectively in the operations of this food committee of ISO. Fortunately, there was considerable overlap in personnel between ISO/TC 34/SC 5 and the analytical methods committee of IDF.

This paper was presented at the Symposium on Food Legislation and Regulation Around the World, sponsored by the Agricultural and Food Chemistry Division, American Chemical Society, Aug. 26, 1975, Chicago, IL.

Received May 18, 1976.

The *Association of Official Analytical Chemists (AOAC)*, composed of scientists chiefly from North America but with members from other countries as well who supply governments with validated methods of analysis for use in regulatory situations. *Official Methods of Analysis* of AOAC (1), which enjoys a world-wide reputation as containing only methods of analysis of proven reliability, includes a chapter on dairy products.

Scientifically, the situation regarding methods for dairy products was fairly simple, since the Roese-Gottlieb method in its various forms was the standard reference method throughout the world for the determination of fat. Fat, of course, is still the most important economic constituent of milk and its products. Politically, the situation was rather difficult because none of the organizations had a very strong reason for wanting to cooperate with the others, except that both IDF and ISO did want the prestige of United States participation. At the urging of the Codex Secretariat, who probably applied some subtle diplomacy, a joint IDF/ISO/AOAC group was formed with the responsibility of supplying jointly approved methods of analysis for supporting international Codex standards for milk and milk products. Within a surprisingly short time, about a dozen methods were approved by the joint group and by their sponsoring organizations and supplied to the Committee of Government Experts. They are tabulated below:

Sampling
general
liquid milk products
condensed and evaporated milk
butter
cheese
Salt in butter
Refractive index of fat from butter
Fat (Roese-Gottlieb method)
milk
evaporated milk
sweetened condensed milk
dried milk
cheese
Acid value of fat from butter

This Committee has been operating smoothly, although progress has been slow, because of the time and distances between meetings. But this year the group recommended methods for the determination of chloride, citrate, and phosphate in processed cheese products. An interlaboratory collaborative study performed by the AOAC showed that the chloride and citrate methods were satisfactory, but the phosphate method was returned for further work because the variability among laboratories was too great. This analytical group is working harmoniously and productively. The only deficiency from the AOAC side is a lack of sufficient volunteers to keep up our end of the structure.

One unexpected by-product of this cooperation on milk products was a similar venture between the AOAC and the International Office of Cocoa and Chocolate (OICC). Both of these organizations had developed, independently, methods of analysis for cocoa products. As a result of serving on the IDF/ISO/AOAC/Codex group, members from both organizations realized the desirability to develop and approve jointly the methods of analysis required by the Codex Committee on Cocoa and Chocolate. Starting with methods for the preparation of samples and Kjeldahl nitrogen in 1969, both organizations now have identical methods for the following additional determinations: ash, pH, fat, and alkalinity of insoluble and soluble ash. In most cases, the best features of the OICC and the AOAC methods were combined into a single method, and then a collaborative study was performed, utilizing collaborators from both organizations. This voluntary arrangement is an outstanding example of what can be accomplished through willing cooperation.

Another effective cooperative arrangement developed independently of the Codex between the groups in the International Union for Pure and Applied Chemistry (IUPAC) and in the AOAC dealing with aflatoxin methods. Although initially IUPAC insisted upon conducting its own collaborative study, they finally saw the wisdom scientifically and economically of utilizing a single international collaborative study as the basis for the adoption by both organizations. Currently, 5 identical methods for aflatoxin in various commodities have been endorsed by both organizations. Uniform AOAC/IUPAC methods also exist for the determination of polynuclear hydrocarbons.

These examples show that uniformity can be achieved on an international basis for methods

of analysis. The following is a discussion of some of the difficulties encountered along the way. By revealing these problems, the issues can be clarified and understood. This is the precursor to their solution.

Semantics.—One of the most frequent points of misunderstanding involves the use of the term "collaborative." In AOAC terminology, this term means the analysis *as unknowns* of a number of samples by a group of laboratories by a defined method for the purpose of determining the reliability of that method. In many other countries and with numerous other organizations, the term "collaborative" means what we would call "cooperative." The distinction is that, in a collaborative study, many laboratories analyze the same samples, thus providing what the statisticians call the "between-laboratory" and "within-laboratory" variances or error terms; in a cooperative study, many laboratories do not analyze the same samples. They provide their own samples and often their own methods or variants of the provided method. At best, these studies can provide only a "within-laboratory" variance or error term.

Therefore, when many of our foreign colleagues are asked if their organizations sponsor collaborative studies, their reply is invariably yes. They are thinking in terms of their rules, which require several rounds of comments by members who may try the method in their laboratories. Unfortunately, their rules do not usually require any laboratory testing prior to final approval.

AOAC is attempting to solve this problem by referring to our type of studies as "interlaboratory collaborative studies." We are pleased to note that many more truly interlaboratory collaborative studies are being performed by other organizations and in other countries. This has been stimulated by the Codex criterion for selection of methods of analysis, which indicates a preference for those methods whose reliability has been established in comparative or collaborative studies in several laboratories.

Decisions.—One sampling plan has already been endorsed by the Codex Alimentarius Commission and submitted to governments for acceptance (2). It applies to processed fruits and vegetables for quality characteristics such as color, texture, defects, and size, and not for factors which present a hazard to health, such as residues and contaminants. The characteristic of this plan is that it sets an acceptable quality level (AQL) of 6.5%. That is, it is a sampling plan which will result in the acceptance of 95% of submitted lots with an average defect level of 6.5%. Some countries have objected to such a plan as not being in the interest of consumers but rather being a plan for the control of manufacturing operations.

But the current sampling plan being debated at both the national and international levels concerns the relatively simple concept of net weight. The apparent simplicity, however, is deceptive. To the consumer, net weight means that every package should contain at least the amount specified on the label. He thinks that his definition is met if 100 lb of sugar is packaged into 100 equal 1 lb boxes. If it is pointed out to him that modern packaging machines are not absolutely accurate, he might be willing to tolerate small deviations as long as the average is 1 lb and the deviations are not excessive. This is known as the *average net weight concept*. But when the concept is put in terms of money, it requires reconsideration. What if your bank, in the interest of economy, started to package its coins in rolls prepared according to the normal frequency distribution curve and you were on the receiving side of the negative half of the curve? You would quickly become a convert to the *minimum net weight* theory which requires every package to have at least the stated net weight! The average net weight theory probably results in small benefits to the consumer in the long run in that less sophisticated packaging equipment can be used, but the minimum net weight concept is much easier to enforce. The decision is not scientific or statistical; it is economic, social, or political. The statisticians can supply statistical sampling plans very quickly, as soon as the decision makers provide an answer to the question of which net weight concept will be adopted.

Untried Methods.—The United States government agencies would like to use, as far as possible, methods developed and approved by other countries or organizations. This is efficient management by permitting each organization to concentrate on those problems of greatest importance to each. However, there must be some assurance that the mechanism used provides practical and reliable methods. Several examples

can be cited to show that the ISO mechanism has some flaws. In one case, ISO had developed a method for the determination of mineral impurities, involving sedimentation in a separatory funnel and drawing off the heavy particles and leaving the light, unwanted plant tissue floating in the liquid. The method did not prove practical, since the heavy particles remained in the stopcock during attempts to draw them off (3). It was apparent that the method had never been tested before its endorsement.

In another case, a typographical error in an important reagent concentration survived many rounds of comments and approval until someone actually tried the method as written and found it inoperable.

The United States solution to this problem is that we do not accept any method unless it has been subjected to an interlaboratory collaborative study which demonstrates its practicality and reliability.

Occasionally when international organizations take over interlaboratory collaborative studies they become overenthusiastic. They put into the study aspects of method development which should have been part of the original laboratory work. On 2 occasions, FDA has had to protest to subsidiary bodies of IUPAC because our laboratory wasted several months on methods which proved to be unsatisfactory. This would have been discovered had the initiating laboratory performed the analysis by the submitted directions before shipping the samples. In one case, they even had comments which indicated that the method would not be satisfactory. Apparently the internal politics of large international organizations requires preparing methods by Committees, which sometimes results, unwittingly, in incompatible requirements.

Fortunately other organizations are seeing the wisdom of conducting interlaboratory studies. They should not repeat our past mistakes. Interlaboratory collaborative studies should be conducted as the end result of analytical research and not at the beginning as a fishing expedition. Research on the optimum operating conditions, such as a Youden ruggedness test (4), should be performed by the initiating laboratory. A fundamental rule of interlaboratory studies should be that the developer of the method should not impose his basic research upon his collaborators.

The AOAC recommends that, when a method is ready for an interlaboratory study, the final written version be tested by someone in the laboratory who did not participate in the previous work. If he has any difficulty with it, further work should be done to clear up the difficulty so that other laboratories are not requested to invest their valuable time in a study which is not likely to lead to a successful conclusion.

Fishing Expeditions.—It is hard to understand the enthusiasm of laboratories to participate in the examination of a set of samples just because they are sent out by a prestigious laboratory (including the Food and Drug Administration's (FDA)). They often have little hesitation in turning down a well planned unglamorous study in the same area. Very recently, many countries accepted an invitation from Australia to participate in the examination of some food samples for pesticide residues under the auspicies of the Codex Committee on Pesticide Residues. The laboratory could use any method it desired. FDA declined to participate, since we already knew that the FDA multiresidue pesticide method (the famous Mills procedure) (5) which we would have used has an overall average recovery of 95% and a relative standard deviation of 15%. It can also be predicted that a free wheeling study such as this will show variations from laboratory to laboratory of several orders of magnitude. Similar studies have been published not only for pesticide residues but for aflatoxin and methyl mercury. The conclusion will be that better methods are needed. Such studies usually ignore the more important aspects of laboratory quality assurance and individual analyst performance. It is also known that the Florisil adsorbent is the critical reagent in the FDA pesticide method. Unless the correct grade, as verified in the laboratory by actual separations, is used, no amount of experience, quality assurance, or interlaboratory studies will provide the correct results.

Such studies can provide useful information under 2 conditions: (*1*) A known sample should be included with a strong recommendation that work should not proceed on the unknown samples unless the results check the given values within, say, 20%; and (*2*) all laboratories should return with their results a description of the method used, so that an at-

tempt can be made to correlate high and low values with operational details. Laboratories which do poorly in such studies should undertake an examination of their method and operations.

Multiple Methods.—In several cases in AOAC work with ISO, we have objected to their incorporation of a number of alternative modifications in such basic methods as crude fiber and Kjeldahl nitrogen. Despite the fact that we have pointed out that some of these variations produce significantly different results, we have been told that these variations are necessary in the interest of harmonizing different points of view. Without these variations, the method would be rejected when brought to a vote or, if it were to be accepted, it would never be used, which would not improve the cause of international standardization.

Although this concept may be politically viable, it is scientifically unacceptable. Because Poland has prohibited the use of mercury in Kjeldahl nitrogen determinations, it does not follow that results by a copper catalyst are equivalent, when the scientific facts are otherwise. The Codex has handled this problem by providing only for "Referee methods," intended for use in case of disputes. Other methods may be used for routine inspection or other control purposes. Methods proven to be equivalent to the Referee methods may be adopted as alternative methods. Equivalent methods are expected to provide for differences in equipment and sophistication from country to country, as for example, alternative colorimetric and atomic absorption methods for trace amounts of toxic elements, volumetric and potentiometric methods for chlorides, and gas chromatographic and thin layer chromatographic methods for pesticide residues.

Overelaborate Methods. — The admirable United Kingdom book *Official, Standardised, and Recommended Methods of Analysis* (6) of the Society for Analytical Chemistry devotes almost 8 pages to the determination of dirt in milk. It is a beautifully elaborate method, requiring an initial sedimentation period of 72 hr, followed by a repetition of the treatment for an additional 48 hr to "disentangle the dirt" from the fat fraction. The dirt is then measured volumetrically in a standard centrifuge tube. The method solemnly declares, "The use of centrifuge tubes tested and approved by the National Physical Laboratory is recommended." It is highly unlikely that any Public Health Official would want to wait at least 5 days to inform a farmer that there was manure in his milk. Contrast this test with the very rapid "sediment test" used in the United States and Canada, which presents immediately to any interested spectator the visible evidence of the dirt in the milk. For developing countries, the use of overelaborate tests will not assist in the production of cleaner milk.

A more recent and sophisticated example is provided by the action of the Codex Committee on Methods of Analysis and Sampling at their 1973 meeting. The Committee recommended for consideration (not for approval) by governments a mass spectrometric method for the detection of adulteration of fruit juices, based on the ratio of ^{18}O to ^{2}H. Plants tend to concentrate the heavier isotopes so that dilution with ordinary water is readily apparent (7). FDA reviewed the resources required to implement this recommendation and found it impractical within our present priorities. Other laboratories also stated that they did not have the resources to study this elegant and interesting method of analysis.

Lack of Adequate Standards or Reference Materials.—The United States has considerable amounts of local currencies in a number of countries that can only be used for cultural, educational, and scientific purposes within that country. The U.S. Department of Agriculture, and to a lesser extent some of the constituent agencies of the Department of Health, Education, and Welfare, have a number of active scientific programs in these countries called "PL-480" projects that utilized these funds for payment of salaries and expenses. One of these contracts with the FDA was intended to verify the reported high values for the contaminants lead, cadmium, copper, and zinc in the food produced in an area of smelting and metallurgical activities. The first values reported under the contract verified the initial data. But after the contractor obtained suitable atomic absorption equipment, the laboratory substituted atomic absorption methods for the former colorimetric methods, utilized FDA-validated methods of analysis, with particular attention to background correction, and employed National Bureau of Standards Standard Reference Mate-

rials in the series. It was found that the levels of lead and cadmium were reduced by as much as a factor of 10. The final conclusion was that the levels of metals in food in this industrial area do not differ significantly from those of other countries. What looked like an interesting epidemiological investigation became a routine analytical surveillance operation.

Similar situations have arisen with respect to other important contaminants in the food supply. Inordinately high values for nitrosamines have been reported in such unlikely foods as wheat cooked at a high temperature, undoubtedly due to the use of nonspecific colorimetric methods. Polynuclear hydrocarbons of several orders of magnitude greater than those found in Western countries had been reported from the Eastern-bloc nations, probably because of the inability of these countries to obtain purified hydrocarbon solvents or efficient adsorbents to prepare such solvents, which are essential to the proper isolation of this class of compounds. The reliability of aflatoxin assays in many parts of the world suffers from the use of unsuitable standards, although in this case, the values are often lower than the true values.

Publication of Results.—A number of international organizations have indicated that they do perform interlaboratory collaborative studies but do not publish the results. The Codex Committee on Methods of Analysis and Sampling requested the International Committee on Uniform Methods of Sugar Analysis (ICUMSA) to supply it with the results of their collaborative studies. Upon investigation, ICUMSA indicated that they were no longer available. Similarly, the Nordic Analytical Committee does not publish the results of its studies. Both ICUMSA and the Nordic Analytical Committee have excellent compilations of methods, which are flawed by the unavailability of published data on which they are based.

In the United States, the AOAC has been unique in publishing the results of its interlaboratory studies since its inception in 1884. Since the trend in governmental operations is toward increasing openness, organizations will have to release the basis of their actions if they expect to have governments utilize the results of their activities.

Microbiological Methods.—Another interesting aspect of coordination is being conducted in microbiology. Here again, the driving force is the Codex Alimentarius Commission's Committee on Food Hygiene. This Committee is preparing draft Codes of Practices for various commodities which include end product microbiological specifications. Utilization of these specifications will require international agreement and uniform application of appropriate methodology for those microbial groups included in the final recommendations.

A number of organizations have already been addressing themselves to the problem of uniform methodology in microbiology from various viewpoints. One of the most important is the *International Commission on Microbiological Specifications for Foods*, a standing Commission of the International Association of Microbiological Societies. It was formed in 1962 in response to the need for internationally acceptable and authoritative decisions on microbiological limits for food commensurate with public health safety, and particularly for foods in international trade. This organization has produced 2 volumes entitled *Microorganisms in Foods*. The first, now under revision, is subtitled "Their Significance and Methods of Enumeration" (8). The second, which has just appeared, is subtitled "Sampling for Microbiological Analysis: Principles and Specific Applications" (9). The United States and Canada have contributed substantially both in personnel and resources to the operations of this Commission.

The dairy industry was the first component of the food business to recognize the importance of microbiology and the necessity for sanitary standards to safeguard public health. The IDF, therefore, had considerable experience in providing standardized procedures for the examination of dairy products. Unfortunately, the specialized nature of this organization and the lack of participation of the United States have limited the influence of this important organization.

Recently, the Technical Committee on Agricultural Food Products of the ISO has established a subcommittee on microbiology, designated as ISO/TC 34/SC 9. All of the subordinate units of this technical committee are eager to assist the Codex Alimentarius in the establishment of acceptable, uniform methods of analysis. The elaborate approval structure of ISO and the fact that the United States regula-

tory agencies such as the Department of Agriculture and the FDA have been unable to participate in the activities of ISO has worked against a more active role for this organization.

AOAC has shown that our mechanism of validation of methods through interlaboratory collaborative studies is applicable to microbiological, biological, and even toxicological methods. These methods are utilized by those United States government agencies which must prove their allegations to a court of law.

In the United States, the methods requirements of the government agencies in relation to the international organizations have been coordinated through a recently established AOAC committee. By this means it is hoped that the United States can speak with a single voice with regard to its microbiological requirements when cooperating with other international organizations.

References

(1) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC

(2) Codex Alimentarius Sampling Plans for Prepackaged Foods (1969) Food and Agriculture Organization/World Health Organization, Rome, Italy, AQL 6.5, CAC/RM 42-1969

(3) Winter, F. W. (1971) *JAOAC* **54**, 581–583

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

(5) *Pesticide Analytical Manual* (1976) Vol. 1, Food and Drug Administration, Washington, DC

(6) Hanson, N. W. (Ed.) (1973) *Official, Standardised, and Recommended Methods of Analysis*, 2nd Ed., The Society for Analytical Chemistry, London, England

(7) Bricout, J. (1973) *JAOAC* **56**, 739–749

(8) Thatcher, F. S., & Clark, D. S. (1968) *Microorganisms in Foods*, Vol. 1, *Their Significance and Methods of Enumeration*, University of Toronto Press, Toronto, Ontario, Canada

(9) International Commission on Microbiological Specifications for Foods of the International Association of Microbiological Societies (1974) *Microorganisms in Foods*, Vol. 2, *Sampling for Microbiological Analysis: Principles and Specific Applications*, University of Toronto Press, Toronto, Ontario, Canada

STATISTICAL TECHNIQUES

Simplex Optimization as a Step in Method Development

THOMAS J. DOLS and BERNARD H. ARMBRECHT
Bureau of Veterinary Medicine, Food and Drug Administration, Rockville, MD 20852

In the course of method development, an optimization procedure should be performed before critical performance characteristics are measured. Interaction among the operational steps should be assumed to exist. The analysis of variance or the simplex procedures are designed to determine defined optimal responses. Other things being equal, the simplex method is more efficient in terms of the number of experiments required. This is due, in part, to the sequentially operated feedback strategy employed. Decisions are made according to 5 defined rules.

Many procedures have been described whereby the functions of a system can be optimized. The least efficient method for assessing the pertinent variable is by the random access, arbitrary choice procedure. A more systematic strategy is to hold all variables or factors except one constant during the investigation for maximizing a defined response. Use of this method introduced a major improvement. Finally, the concept for judging a system in which factors can interact was reported by Fisher (1) in 1935. The univariant and the multivariant modes continued to be used and improved for several years.

Fisher advocated the use of a factorial design, with the results to be treated using his analysis of variance method. The number of experiments or samples required is 2^n, where n is the number of factors studied. All experiments need to be conducted simultaneously. (This plan assumes that all experiments vary in the same way. In an analytical method development, the statistical control factor will cancel out.) This requirement was easily met in agricultural applications where a large number of land plots, planted with a crop, were studied.

In analytical applications, the use of fractional factorials, incomplete block designs, the Youden square, a Latin square, or nested designs can be considered (2). In engineering practice, 2-level factorials, as described by Brownlee (3) and Davis (4), or the simplified versions reported by Davies (4), Box and Hunter (5), or Adelman (6, 7), are well established.

The simultaneous analysis of a large number of chemical samples by manual methods presented problems of execution that gave impetus to the univariant school. In 1941, Hotelling (8) described some systematic methods for finding an optimum condition. Friedman and Savage (9) suggested a sequential search modification for the single factor model that was later proved to be valuable.

Finding an optimum solution from among a large set of possibilities is the function of operations research (10). In addition to those strategies described above, factor analysis was developed as a means to recognize patterned, interacting relationships present in large matrices of data found in columns and rows, for which a FORTRAN IV computer program is available (11). Another attractive strategy which can be used to find an optimum solution is the simplex method. Spendley *et al.* (12) derived the theoretical principles for the sequential simplex method that amalgamated the desirable features of allowing for factor interaction and sequential examination. This evolutionary model was reduced to practice by 2 derivative procedures, namely, the Long method (13, 14), as extended by Czech (15, 16), and that of Deming and his students (17–19). Deming has developed a computer program which is convenient when a large number of factors are examined simultaneously (S. N. Deming, 1975, Department of Chemistry, University of Houston, Houston, TX 77004).

The simplex concept met with early acceptance by the chemical engineering community (20–24), in the optimization of instrument per-

This paper was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Received August 15, 1975.

formance (25), and in application to analytical chemical method development (13–19, 26–29). In this paper, the simplex method is outlined.

Simplex Rules

A simplex is a figure containing $n + 1$ vertices, where n is the number of factors studied. Thus, when 2 factors are studied and plotted, a triangle results and a tetrahedron is obtained for 3 factors. More than 3 factors cannot be illustrated graphically. An analytical solution by tabulation (13–16) or by computer is utilized.

The initial location point of a triangular simplex can be deduced from exploratory work on a method. Limits on the factors examined (called constraints or boundaries) need to be identified. The rules include provisions when the boundaries are exceeded. The step size of the simplex triangle sides should be more than 3 standard deviations of the method's precision, otherwise, the movement of the simplex will be erratic. By way of illustration, a 2-factor, equal step size hypothetical example is given in Table 1. The first 3 experimental values define vertices 1, 2, and 3. This is the function called for in the first rule.

Rule 1: A move is made after each observation of response (17).

To continue the operation, the factor values for vertex 4 can be calculated as shown in Table 2 or as given by Deming and Morgan (17) in the next rule.

Rule 2: A move is made into that adjacent simplex which is obtained by discarding the point of the current simplex corresponding to the least desirable response and replacing it with its mirror image, the (hyper) face of the remaining points (*see* Table 1 where vertex 1 is discarded).

The illustration (Table 1) shows a simplex stranded on a ridge in space. The next rule (17) is designed to remedy this problem.

Rule 3: If the reflected point has the least desirable response in the new simplex, do not reapply Rule 2, but reject the second lowest response in the new simplex and continue (in Table 1, vertex 2 is discarded).

Occasionally, an erroneous result is obtained (the method precision may not be in control) and the next rule is applied (27).

Rule 4: If a vertex has been retained in $k + 1$ simplexes, where k is the previous sum of simplexes examined, rerun the experiment before continuing.

The abnormal result probably will not be verified and the redetermined value should be used. If the calculation of factor values for a new vertex by Long's method (13, 14) or the flipover maneuver of Deming falls outside the constraints for factor values, the next rule is applied (17, 27).

Rule 5: If the calculated coordinates at the new point lie outside the boundaries of one or more variable factors, assign an undesirable value and continue.

Then, an application of Rules 2 and 3 will force the simplex back into appropriate limits.

At an optimum, the rules force the simplex to circle the optimum point. In order to verify an optimum, a new simplex can be started at another location. If this progression moves to the previously found optimum, the level of confidence is increased (*see* ref. 27, for example). The main obstacles that might exist are long ridges or saddle formations in space. In such cases, isosceles, right, or oblique triangles may be substituted for equilateral triangles.

If more than 2 factors are studied, Long's tabulation method (13–16) is suggested. While a tetrahedron simplex can be displayed graphically, it will not give a close-pack at optimum. Other strategies and details can be found in the papers of Czech (15, 16), Deming (17–19), or Nelder and Mead (30).

Merits of Simplex Optimization Method

A few studies in which simplex optimization (SO) was conducted vs. the regular method are summarized in Table 3, using sensitivity enhancement as the measured response. Czech (15, 16) conducted comparative studies of SO methods for sugar products.

Table 1. Simplex factors[a]

Vertex No.	Factor A	Factor B	Response
1	10	45	0.5
2	10	55	0.9
3	20	50	1.2
4	20	60	0.7
5	30	55	1.4

[a] Source: Krause and Lott (27).

Table 2. Coordinate calculation of a new vertex[a]

Operation	Vertex No.	Factor A	Factor B
Retained vertices	2	10	55
	3	20	50
Sum		30	105
Coordinate × 2/n			
Less coordinates of discarded vertex	1	−10	−45
Coordinates of new vertex	4	20	60

[a] Sources: Long (13, 14) and Krause and Lott (27).

Table 3. Simplex optimized methods

Method of analysis	Enhancement of sensitivity	Ref.
Sulfur dioxide by *p*-rosaniline	7	13
Phosphate by molybdenum blue	13	13
Formaldehyde in sugar products by AOAC J-acid method	5	15, 16

(*1*) The J-acid SO method was 5 times more sensitive than the non-optimized method.

(*2*) The SO acetylacetone method was about 4 times more sensitive than the non-optimized AOAC method.

(*3*) The sensitivity of the acetylacetone method vs. the J-acid method was improved 33%, or vs. the chromotropic acid method by 45%.

Czech and others found the time required by an analyst to conduct an analysis was substantially reduced.

Some disadvantages can also be listed.

(*1*) The sequential analyses used in carrying out an SO evaluation need to be in "statistical control," where all causes of error have been measured.

(*2*) Multiple factor analyses may not provide a precise definition of the optimum conditions. However, the 2 dominant factors can be further evaluated by the 2-factor simplex method.

Some modifications of the rules are listed above and it is possible that other problems may be discovered when more experience with the method is gained.

Comment

A multivariate, interacting complex of factors in an analytical method should be presumed until proven otherwise. The analyst can elect to use one of the various factorial models as reviewed by Koehler (31) or consider the sequential simplex method. In rare instances when no interaction among factors is present, these alternative method choices will not be prejudiced. However, the newly developed uniplex method of King and Deming (19) can be examined for applicability to the situation at hand.

It is suggested that an optimization procedure be introduced into the method development plan preferably during the preliminary feasibility phase and certainly before the performance characteristics of the method are finally evaluated. While the simplex method is relatively new, the experience found to date is very favorable, and the procedure warrants serious consideration as a method of choice.

References

(1) Fisher, R. A. (1935) *The Design of Experiments,* Oliver and Boyd, Edinburgh, Scotland

(2) American National Standard K 65.50 (1971) American National Standards Institute, New York, NY

(3) Brownlee, K. A. (1953) *Industrial Experimentation,* Chemical Publishing Co., New York, NY

(4) Davies, O. L. (Ed.) (1960) *Design and Analysis of Industrial Experiments,* Hafner Publishing Co., New York, NY

(5) Box, G. E. P., & Hunter, J. S. (1961) *Technometrics* **3**, 311–351

(6) Adelman, S. (1962) *Technometrics* **4**, 21–46

(7) Adelman, S. (1962) *Technometrics* **4**, 47–58

(8) Hotelling, H. (1941) *Ann. Math. Stat.* **12**, 20–45

(9) Friedman, M., & Savage, L. J. (1947) in *Techniques in Statistical Analysis,* Eisenhart, C., Hastay, M. W., & Wallis, W. A. (Eds.) McGraw Hill, New York, NY

(10) Massart, D. L., & Kaufman, L. (1975) *Anal. Chem.* **47**(14), 1244A–1257A

(11) Howery, D. G. (1976) *Am. Lab.* **8**(2), 14–25

(12) Spendley, W., Hext, G. R., & Himsworth, F. R. (1962) *Technometrics* **4**, 441–461

(13) Long, D. E. (1969) *Anal. Chim. Acta* **46**, 193–206

(14) Houle, M. J., Long, D. E., & Smette, D. (1970) *Anal. Lett.* **3**(8), 401

(15) Czech, F. P. (1973) *JAOAC* **56**, 1489–1495

(16) Czech, F. P. (1973) *JAOAC* **56**, 1496–1502

(17) Deming, S. N., & Morgan, L. L. (1973) *Anal. Chem.* **45**(3), 278A–283A

(18) Morgan, S. L., & Deming, S. N. (1974) *Anal. Chem.* **46**, 1170–1181

(19) King, P. G., & Deming, S. N. (1974) *Anal. Chem.* **46**, 1476–1481

(20) Baasel, W. E. (1965) *Chem. Eng.* **72**(22), 147–152

(21) Carpenter, B. H., & Sweeney, H. C. (1965) *Chem. Eng.* **72**(14), 117–126

(22) Kenworthy, I. C. (1967) *Appl. Stat.* **16**, 211–224

(23) Lowe, C. W. (1964) *Trans. Inst. Chem. Eng.* **42**, T334

(24) Lowe, C. W. (1967) *Trans. Inst. Chem. Eng.* **45**, T3

(25) Ernst, R. R. (1968) *Rev. Sci. Instrum.* **39**, 998–1012

(26) Dauwe, C., Dorikens, M., & Dorikens, L. (1974) *Van Praet Appl. Phys.* **5**, 45–47
(27) Krause, R. D., & Lott, J. A. (1974) *Clin. Chem.* **20**, 775–782
(28) Veksler, M. A., Karpova, L. K., & Shemyakin, F. M. (1972) *Zavod. Lab.* **38**, 333–334
(29) Wokrdj, A., & Wokrdj, A. (1973) *Przem. Chem.* **52**, 227
(30) Nelder, J. A., & Mead, R. (1965) *Comput. J.* **7**, 308–312
(31) Koehler, T. L. (1960) *Chem. Eng.* **67**(25), 142–152

New Statistical Manual

The *Statistical Manual of the AOAC,* a 96-page booklet, is a composite, with revisions, of the popular AOAC monograph, *Statistical Techniques for Collaborative Tests,* by the late W. J. Youden, and a companion volume, *Planning and Analysis of Results of Collaborative Tests,* written by E. H. Steiner and originally published in 1974 by the British Food Manufacturing Industries Research Association, who have assigned their copyright to the AOAC. This book is intended for use in the statistical analysis of the results of interlaboratory collaborative tests, such as those required by the AOAC before its adoption as official of analytical methods for agricultural products, foods, beverages, drugs, cosmetics, color additives, and other commodities important in public health.

The Youden monograph was specifically written as a "do-it-yourself" manual for those who are at home in the analytical laboratory but who have little or no experience with formal statistics. It presents simple and flexible statistical techniques, using examples related to familiar questions concerning the significance of apparent differences among results. The Steiner manual, intended for those who are already familiar with the fundamentals covered by Youden, presents guidelines for the planning of collaborative tests and introduces the analysis of variance technique with examples of how it may be used in the analysis of interlaboratory tests.

Order from the Association of Official Analytical Chemists, P.O. Box 540, Benjamin Franklin Station, Washington, DC 20044; price $5.50, including postage and handling. Please enclose remittance with orders under $10.00.

FOOD ADDITIVES

Rapid Determination of Butylated Hydroxyanisole, *tert*-Butylhydroquinone, and Propyl Gallate in Edible Oils by Electron Capture Gas-Liquid Chromatography

B. DENIS PAGE and BARRY P. C. KENNEDY
Food Research Division, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario, Canada K1A 0L2

A rapid method is described for the determination of 2- and 3-*tert*-butyl-4-hydroxyanisole (2- and 3-BHA), *tert*-butylhydroquinone (TBHQ), and propyl gallate (PG) as their heptafluorobutyrates in edible oils. A solution of the oil in ether-benzene is treated with heptafluorobutyric anhydride (HFBA) in the presence of trimethylamine as catalyst. Excess HFBA is destroyed and a suitably diluted aliquot is analyzed by electron capture gas-liquid chromatography. An internal standard is added before derivatization to aid quantitation and identification of the antioxidants. Average recoveries from an oil spiked at 1, 5, 5, and 5 ppm TBHQ, 2-BHA, 3-BHA, and PG were 110, 105, 106, and 102%, respectively. At these low levels, however, some late-eluting peaks interfered. Butylated hydroxytoluene is not determined by this method.

Phenolic antioxidants are generally used to prevent oxidative breakdown and thus improve the flavor stability of fats and oils. In Canada, butylated hydroxyanisole (BHA, a mixture of 2- and 3-*tert*-butyl-4-hydroxyanisole), butylated hydroxytoluene (BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene), and propyl gallate (PG, propyl-3,4,5-trihydroxybenzoate) are permitted either singly or in combination in fats and oils at levels up to 200 ppm. Nordihydroguaiaretic acid (NDGA), a naturally occurring antioxidant, and *tert*-butylhydroquinone (TBHQ), a recently developed antioxidant, are not permitted for food use in Canada.

In recent years, a variety of gas-liquid chromatographic (GLC) techniques for the estimation of the above phenolic antioxidants have been developed. However, in most of these methods, time-consuming isolation procedures such as liquid-liquid extraction (1–3), column chromatography (1, 3–5), sublimation (6, 7), or fat precipitation (8) are required before the final analysis. In those methods, only BHA and BHT have been successfully chromatographed without prior derivatization (6). Thus, diluted oils containing these 2 compounds may be injected directly onto a precolumn containing glass wool (8). However, this procedure necessitates daily cleaning of the precolumn.

Precolumns or lengthy cleanup procedures can be avoided and low levels of the phenolic antioxidants can be estimated rapidly by derivatizing the antioxidant, which increases the sensitivity to detection by electron capture chromatography. Perfluoroacylation, particularly with heptafluorobutyric anhydride (HFBA), trimethylamine as catalyst, and benzene as solvent, has been successfully applied to determine nanogram or picogram quantities of phenolic compounds (9, 10). The derivatization procedure is both rapid and quantitative.

This paper describes the application of derivatization, direct injection of the diluted oil, and electron capture GLC to the rapid determination of 2- and 3-BHA, TBHQ, and PG as their heptafluorobutyrates in vegetable oils.

METHOD

Apparatus

(a) *Gas chromatograph.*—Varian Aerograph Model 2100, or equivalent, with Sc^3H electron capture detector and 6′ × 2 mm id U-shaped glass column packed with 3% OV-3 on 80–100 mesh Chromosorb W (HP). Operating conditions: injector 200°C, column 120°C, detector 250°C, nitrogen carrier gas flow 20 ml/min. Inject all solutions by using ca 1.5 μl hexane as solvent flush. Adjust range and attenuation settings so that ca 200 pg 2,3,4,5-tetrachlorophenyl heptafluorobutyrate (TCP-HFB) prepared as described under *Derivatization* gives 50–80% full scale deflection. Retention time for TCP-HFB should be ca 4.5 min.

(b) *General glassware.*—Treat all glassware with 10% (w/v) methanolic KOH for 2 hr, rinse well with methanol, dry, and silanize overnight with

dichlorodimethylsilane-toluene (20+80). Rinse with toluene, methanol, and acetone and dry in 110°C oven. To re-use silanized glassware, rinse well with acetone and blow dry with stream of dry nitrogen.

Reagents

(a) *Solvents.*—Distilled-in-glass grade hexane and benzene. Reagent grade absolute ether containing no added ethanol or other preservative.

(b) *Antioxidants.*—Tenox TBHQ, BHA (a mixture of 2- and 3-BHA), and PG (Eastman Chemical Products, Inc., Kingsport, TN), or equivalent; 2-BHA and 3-BHA (Food Chemicals Codex reference standard).

(c) *2,3,4,5-Tetrachlorophenol (TCP).*—Use highest purity available, or recrystallize technical grade (>95%, available from Aldrich Chemical Co.) from hexane so that TCP-HFB derivative, prepared as described under *Derivatization* and injected into gas chromatograph, shows single peak except for trace impurity, with retention time of 0.49 relative to TCP-HFB.

(d) *Heptafluorobutyric anhydride.*—(Available from Pierce Chemical Co., Rockford, IL or PCR Inc., Gainesville, FL). Open ampoules and transfer contents under dry nitrogen, using glove box or bag. Store in 1 ml vials with Teflon-lined screw caps.

(e) *Trimethylamine (TMA) solution.*—0.2*M*. (Anhydrous reagent available from Eastman Chemical Co.) Add chilled contents of glass, sealed bottle (100 g) to tared bottle(s) containing ca 1700 ml chilled benzene to give ca 1*M* solution. Prepare ca 0.2*M* TMA solution by diluting with ether-benzene (1+99).

(f) *Buffer.*—pH 6. Prepare phosphate buffer from 6 ml 0.2*M* Na_2HPO_4 and 44 ml 0.2*M* NaH_2PO_4. Dilute to 100 ml with water.

(g) *Internal standard solutions.*—10 μg/ml. Store all solutions of internal standard and antioxidants under refrigeration and out of direct light. Accurately weigh and transfer 50 mg TCP to 50 ml volumetric flask. Dissolve and dilute to volume with benzene. Pipet 1 ml aliquot into 100 ml volumetric flask, dilute to volume with ether-benzene (1+99), and mix.

(h) *Reference standard solutions.*—(*1*) *Stock standard solutions.*—1 mg/ml. Accurately weigh and transfer 50 mg each of 2-BHA, 3-BHA, BHA (commercial mixture of isomers), TBHQ, and PG to separate 50 ml volumetric flasks. Dissolve and dilute to volume with benzene for 2-BHA, 3-BHA, and BHA (mixed isomers), and with ether for TBHQ and PG.

(*2*) *Mixed standards for preparation of calibration curves.*—Using above stock solutions, accurately prepare mixed standard solutions containing TBHQ, BHA (mixed isomers), and PG at 0.5, 3, and 4 μg/ml, respectively, in ether-benzene (1+99).

(*3*) *Mixed standards for spiking.*—Using above stock solutions, accurately prepare mixed standard solutions containing TBHQ, 2-BHA, 3-BHA, and PG at 2, 10, 10, and 10 μg/ml, respectively, in ether-benzene (1+99).

Preparation of Sample

For general screening of BHA or PG at levels from 50 to 100 ppm, accurately weigh ca 100 mg oil into 10 ml volumetric flask. For TBHQ at this level, weigh ca 20 mg oil. Pipet 0.5 ml internal standard solution (10 μg/ml) into flask, dilute to ca 10 ml with ether-benzene (1+99), and mix. For higher antioxidant levels, weigh less oil.

For antioxidant levels of ca 25 ppm BHA or PG, weigh 400 mg oil. For TBHQ at this level, weigh ca 80 mg oil. Add 0.5 ml internal standard and proceed as above. For levels of antoxidants as low as 1 ppm, 1 g sample weight is necessary.

Derivatization

Place ca 0.5 ml 0.2*M* TMA in glass-stoppered, 5 ml graduated centrifuge tube; use graduations to estimate volume. Under dry nitrogen, in glove box or bag, add ca 20 μl HFBA with syringe. Add ca 0.5 ml solution to be derivatized (containing internal standard) and shake vigorously or mix 1 min on vortex mixer. Let derivatization proceed 5 min. Add ca 1.5 ml pH 6 buffer and shake vigorously or mix 1 min on vortex mixer. Centrifuge briefly to cleanly separate layers. With Pasteur pipet, transfer ca 0.5 ml upper (benzene-ether) layer to glass-stoppered, 5 ml graduated centrifuge tube containing ca 1.5 ml hexane. Add ca 2 ml pH 6 buffer and shake and centrifuge as before. Inject in duplicate 3 μl upper (benzene-ether-hexane) layer onto GLC column, using ca 1.5 μl hexane as solvent flush. Identify peaks by their relative retention times (Table 1).

Preparation of Calibration Curves

Prepare 6 solutions in ether-benzene (1+99) by accurately diluting mixed standard for preparation of calibration curves and internal standard solution (diluted to 1 μg/ml) such that each solution contains TCP at 0.5 μg/ml and TBHQ, BHA (mixed isomers), and PG with concentration ranges of 0.025–0.25, 0.15–1.5, and 0.2–2 μg/ml, respectively. Derivatize as described above and inject ca 3.0 μl in duplicate onto GLC column. Calculate peak height ratios (antioxidant to internal standard) for duplicate injections at each level for each antioxidant, using sum of isomer peaks

Table 1. Retention times, relative to TCP-HFB, and detector response for some antioxidant derivatives on OV-3 column

Antioxidant HFB	Number of HFB groups	Rel. retention time	Sensitivity,[a] pg
TBHQ	2	0.429	15
2-BHA	1	0.608	60
3-BHA	1	0.694	75
TCP	1	1.00[b]	25
PG	3	1.86	150

[a] Picograms necessary to give 10% deflection at 32×10^{-10} amp, 1 mv full scale.
[b] Retention time for TCP-HFB is ca 4.5 min.

for BHA. Duplicate determinations should agree ±3%. Plot peak height ratios against weight ratios (R, antioxidant to standard) and draw curves.

Calculations

Calculate average peak height ratio from duplicate injections of sample and determine R for particular antioxidant from appropriate calibration curve. Calculate ppm antioxidant in oil sample as

$$\text{Antioxidant, ppm} = (W' \times R)/W$$

where $W' = \mu$g internal standard, and $W =$ g oil sample.

Results and Discussion

The Ehrsson *et al.* derivatization procedure (9) was studied by using flame ionization GLC and 1 mg quantities of each antioxidant. Acylation with HFBA in 0.05*M* TMA rapidly and quantitatively converted the antioxidants to their heptafluorobutyrates (HFB). Excess HFBA was removed by aqueous pH 6 buffer. Combined GLC-mass spectral analysis (Hitachi Perkin-Elmer Model RMS-4) showed that mono-HFB derivatives were obtained with 2- and 3-BHA, di-HFB with TBHQ, and tri-HFB with PG. As little as 0.1 μg antioxidant/ml in the diluted derivative solution could be determined readily with the electron capture detector. However, attempts to derivatize the antioxidants at the 0.1 μg/ml level did not give peaks comparable to the diluted derivative. These smaller peaks, especially the PG-HFB, seemed to be due to the low solubility of PG in benzene and its adsorption to glass. Acid- and base-washing of all glassware, followed by silanization, did not fully correct this problem. Adding 1% ether to benzene solutions containing low levels of antioxidants increased the PG derivatization yield to more acceptable and consistent levels. However, the GLC of this ether-benzene solution gave a broad, tailing solvent peak. This interfered with the early-eluting peaks, particularly TBHQ-HFB. To reduce this tailing, a portion of the ether-benzene solution was removed, diluted with hexane, and rebuffered with additional buffer. The 4-fold dilution with hexane was compensated by derivatizing 4 times more antioxidant. These modifications led to the final derivatization procedure. A representative chromatogram is shown in Fig. 1A.

The approximations involved in the dilution of the ether-benzene (1+99) derivative solution for rebuffering necessitates the use of an internal standard. The internal standard also facilitates quantitation and identification of the antioxidants. TCP was chosen as the internal standard because TCP-HFB does not interfere with the antioxidant derivatives. In addition, the absence of underivatized TCP would serve to indicate a complete derivatization reaction for the antioxidants. The phenolic TCP elutes as a broad, tailing peak with a retention time of 1.63 relative to TCP-HFB.

The direct current mode of the electron capture detector is known to have a limited working linear range (11). Calibration plots of peak height ratios (antioxidant to internal standard) against weight ratios (antioxidant to internal standard) for TBHQ, BHA (mixed isomers), and PG were nonlinear with the Sc^3H detector. The sensitivity of the detector to the HFB derivatives of the antioxidants is given in Table 1. For a 1 g oil sample dissolved in 10 ml, a 10% full scale deflection corresponds to ca 0.5, 2.5, 2.5, and 5 ppm TBHQ, 2-BHA, 3-BHA and PG, respectively.

The average recovery values shown in Table 2 were determined by adding a suitable aliquot of standard solution to the 10 ml flask containing the weighed oil. The solvent was then evaporated in a stream of dry nitrogen. The internal standard solution and the ether-benzene (1+99) were added and the sample was derivatized. The peak height ratios from this determination were compared with those from a similar determination in which oil was not added to obtain the per cent recovery. The recovery values shown in Table 2 reflect the interferences from the presence of large amounts of oil in the derivatizing solution and in the sample injected onto the GLC column. Corn oil with no added phenolic antioxidants was chosen for recovery studies.

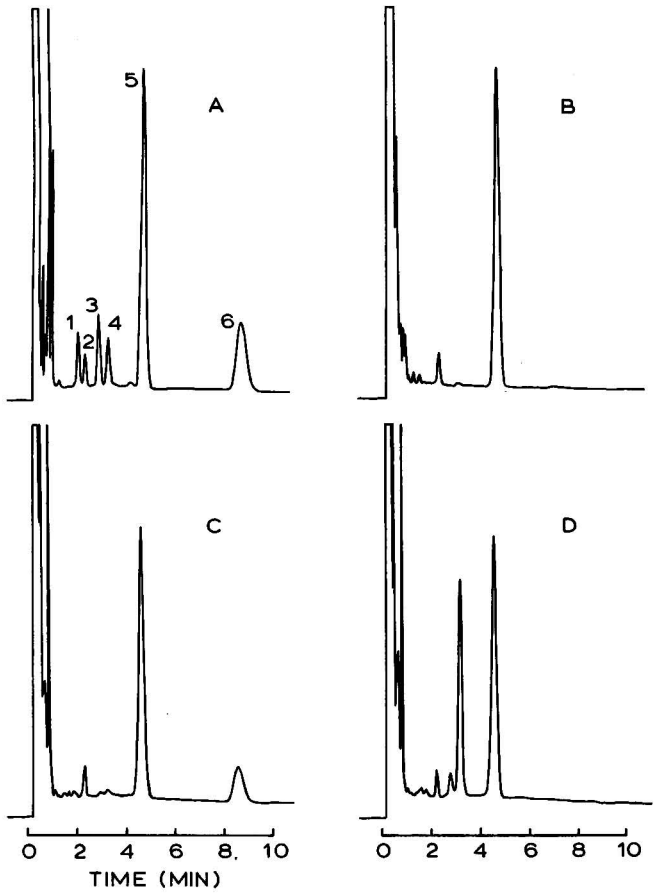


FIG. 1—Gas chromatograms of A, mixed standard: 1, TBHQ-HFB (40 pg); 2, impurity from TCP; 3, 2-BHA-HFB (230 pg); 4, 3-BHA-HFB (230 pg); 5, TCP-HFB internal standard (300 pg); 6, PG-HFB (370 pg); B, corn oil (37 μg oil injected), no detectable antioxidants; C, canbra and soya oil (22 μg oil injected) containing 5 ppm PG; D, soya oil (22 μg oil injected) containing 24 ppm BHA.

Table 2. Recovery of TBHQ, 2-BHA, 3-BHA, and PG from spiked corn oil

Wt of oil, mg	Level of addn, μg/g	Recovery, %			
		TBHQ	2-BHA	3-BHA	PG
100[a]	20	101.1±3.8			
	100		101.4±2.8	102.4±3.8	98.6± 5.5
400[a]	5	99.1±1.6			
	25		95.6±4.0	96.3±5.8	100.6±10.5
1000[b]	1	109.7±5.3			
	5		104.7±7.5	105.8±6.8	101.9±10.8

[a] Average of 4 determinations±std dev.
[b] Average of 6 determinations±std dev.

In the lowest level recovery studies, in which 1 g oil is sampled, broad peaks with retention times relative to TCP-HFB of 2.78 and 4.13 occasionally interfered with subsequent determinations if not allowed to elute before subsequent injections. These interferences were about 10% full scale deflection. An injection of the oil before derivatization did not show such peaks, demonstrating that these peaks resulted from derivatization of oil components. When the analytical procedure is used as a screening method and 100 mg oil is sampled, minimal interference from late-eluting peaks is encountered.

The BHA used by food manufacturers is a mixture of the 2- and 3-isomers, with normal ranges of 5–10% and 90–95%, respectively (12). Thus, for an analysis of commercially available vegetable oil, the sum of the peak heights of the 2 isomers is used to calculate the total amount of BHA present.

Antioxidants determined in some commercially available vegetable oils are presented in Table 3 and some typical chromatograms are shown in Fig. 1. In these determinations, various sample weights were taken to evaluate possible effects of sample size on the antioxidant level obtained. No interferences were found. In all cases, a 150 mg sample or less gave readily measurable peaks.

For a 100 mg sample, a 3 μl injection deposits 3.75 μg nonvolatile oil on the GLC column. With the column used in this study, up to 75 samples have been analyzed without noticeable loss in detector sensitivity or column resolution, and without changes in retention characteristics. With a heavier loading of stationary phase and a larger diameter column, a longer column life would be expected. To minimize column and detector contamination, the column and detector temperatures were increased to 200 and 280°C, respectively, overnight. Operating the electron capture detector at 250°C helps prevent deposition of relatively nonvolatile oil components which may elute from the GLC column. In addition, this detector is insensitive to the major oil components such as the triglycerides. However, it is possible that derivatized oil components may produce extraneous peaks in the area of the antioxidant derivatives. In the oils examined, such interfering peaks were not evident.

The major disadvantage in the described method is its inability to detect BHT. This is undoubtedly because of the steric hindrance of 2 *tert*-butyl groups ortho to the hydroxyl group. In fact, BHT at 1000 ppm does not give any peaks. Despite the lack of response with BHT, the described method is useful for the rapid determination of TBHQ, BHA, and PG at relatively low levels. The average time for a single analysis with one GLC injection from sample acquisition to final result is about 30 min.

Table 3. Phenolic antioxidants found in commercially available vegetable oils, μg/g

Oil	Wt analyzed, mg	BHA	PG
Canbra and soya	149		5
	300		5
	605		4
	593		4
Canbra and soya	98	92	
	159	88	
Corn	1000	[a]	[a]
Corn	1000	[a]	[a]
Soya	303	25	
	591	24	

[a] No phenolic antioxidants found or declared on label.

References

(1) Stoddard, E. E. (1972) *JAOAC* **55**, 1081–1084
(2) Miethke, H. (1966) *Deut. Lebensm. Rundsch.* **62**, 267–270
(3) Schwien, W. G., Miller, B. J., & Conroy, H. (1966) *JAOAC* **49**, 809–812
(4) Groebel, W., & Wessels, A. (1973) *Deut. Lebensm. Rundsch.* **69**, 453–459
(5) Singh, J., & Lapointe, M. R. (1974) *JAOAC* **57**, 804–805
(6) McCaulley, D. F., Fazio, T., Howard, J. W., DiCiurcio, F. M., & Ives, J. (1967) *JAOAC* **50**, 243–250
(7) Fazio, T., Howard, J. W., & Sandoval, A. (1968) *JAOAC* **51**, 17–20
(8) Hartman, K. T., & Rose, L. C. (1970) *J. Am. Oil Chem. Soc.* **47**, 7–10
(9) Ehrsson, H., Walle, T., & Brötell, H. (1971) *Acta Pharm. Suecica* **8**, 319–328
(10) Fenimore, D. C., Freeman, R. R., & Loy, P. R. (1973) *Anal. Chem.* **45**, 2331–2335
(11) Burgett, C. A. (1974) *Res. Dev.* **25**(11), 28–36
(12) Mahn, F. P., Viswanathan, V., Plinton, C., Venturella, V. S., & Senkowski, B. Z. (1970) *JAOAC* **53**, 1179–1181

This paper was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.
Received October 8, 1975.

FEEDS

Pollution-Reduced Kjeldahl Method for Crude Protein

PAUL R. REXROAD and ROBERT D. CATHEY
University of Missouri, Columbia, MO 65201

Pollution from the Kjeldahl method for crude protein has been reduced by substituting a low level of copper (0.04 g $CuSO_4$) for the mercury (0.7 g HgO) specified in the AOAC official method, 2.049. Adjustments were made in the salt-acid ratio so the new system could handle hard-to-digest samples in a reasonable time. The new method was rugged for lysine.HCl. It is designed to be used for crude protein in feeds or similar Kjeldahl work. Precision and accuracy were equal to or better than that for the official method in a study of 17 samples analyzed in duplicate on 3 different days. The following samples were used in the study: lysine.HCl, tryptophan, NBS standards, urea, meals, mixed feeds, grains, and forage. The average per cent nitrogen found was 9.52 by the official method and 9.53 by the copper method. The average standard deviation was 0.038 by the official method and 0.033 by the copper method, giving the corresponding relative standard deviations of 0.40 and 0.35%.

Work has continued in relation to the potential hazards associated with the use of mercury in Kjeldahl work (1–5). This paper brings together the results of experiments on the use of copper, the amount of copper, the salt-acid ratios, and the length of digestion. As reported in the 1972 Associate Referee report, good recovery was obtained for nicotinic acid by using 5 mg $CuSO_4$ with 80 min digestion after clearing (4). Conditions including a salt-acid ratio of 20 g K_2SO_4 to 18 ml H_2SO_4, 30 mg $CuSO_4$, and 90 min digestion after clearing were somewhat "rugged" for 0.5 g nicotinic acid. Some problems encountered with caking at the end of digestion were considered undesirable. The high recovery levels for nicotinic acid with copper as a catalyst led us to believe that the differences in the 1955 collaborative study (6), favoring mercury over copper, could be overcome by selected salt-acid ratios, heat input, etc.

Method Development

The caking encountered at the end of the digestion with the 1:1 or higher salt-acid ratios showed that this would severely restrict the sample size range. Therefore, lower salt-acid ratios were investigated. Modification of the 15 g K_2SO_4–25 ml H_2SO_4 in the current official method, **2.049** (7), was investigated. Experience showed that a 15:20 ratio would reach the higher temperature sooner and still allow enough excess acid for a satisfactory range in sample size.

Information indicated that lysine and tryptophan were the hardest of the protein amino acids to digest (8). These were evaluated by using 1 g lysine.HCl or tryptophan, 15 g K_2SO_4, 20 ml H_2SO_4, 0.1 g $CuSO_4$, and variable digestion times after the dense white fumes cleared from the bulb of the flask. Under these conditions, lysine.HCl proved more difficult to digest than tryptophan, as shown in Fig. 1. With the accumulated information, it was decided that good recoveries for lysine.HCl would be a significant criterion in selecting a final method. Work then proceeded on a mercury-free method for Kjeldahl nitrogen in nitrate-free samples. A primary projected application would be the determination of crude protein in grain, feed, and food samples.

Perrin (9) commented on the use of copper as a catalyst. He stated that $CuSO_4$ in conjunction with zinc in the distillation step resulted in carry-over of NaOH into the receiving flasks. Shedd (10) in 1927 reported similar results. The precipitation of copper with a sulfur source before distillation was recommended. Our preliminary work had not indicated high results for the copper catalyst with zinc as an anti-bumping agent. In a limited study, we did find conditions where the combination of copper and zinc in the distillation step resulted in a significant carry-over of NaOH. We substituted alundum for zinc and had no further problems with NaOH carry-over which were associated with the copper-zinc combination.

This paper was presented at the 88th Annual Meeting of the AOAC, Oct. 14-17, 1974, at Washington, DC.

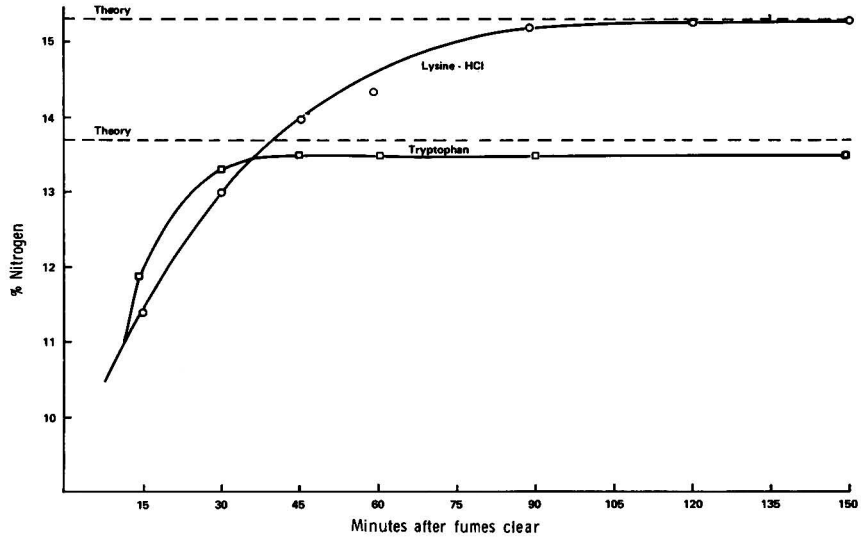


FIG. 1—Digestion of lysine . HCl and tryptophan.

The amount of $CuSO_4$ needed for a 15:20 salt-acid ratio was investigated (Table 1). There was little difference between 5 and 1000 mg $CuSO_4$ as a catalyst. The digestion time was 120 min after clearing. To ensure enough catalyst in each 15 g of commercial blends, a ratio of 40 mg $CuSO_4$–15 g K_2SO_4 was chosen.

In the next study, digestion time and sample size were varied (Fig. 2). Varying the sample size from 0.2 to 1.2 g lysine.HCl had no clear-cut effect. It did indicate that 1.2 g would be acceptable even with a 120 min digestion after clearing. A 15:20 salt-acid ratio was used with 40 mg $CuSO_4$. The digestion time did follow a pattern. Based on these data it was decided to accept a digestion time of 90 min after clearing of the dense, white fumes from the bulb of the flask, pending further evaluation.

Table 1. Amount of $CuSO_4$ vs. nitrogen recovery

$CuSO_4$, mg	Nitrogen,[a] %
0	12.49, 12.76
5	15.16, 15.20
10	15.22, 15.24
20	15.20, 15.24
40	15.25, 15.26
80	15.23, 15.24
100	15.26, 15.23
500	15.22, 15.23
1000	15.20, 15.23

[a] Used 1 g lysine.HCl (theory 15.34% nitrogen), 15 g K_2SO_4, 20 ml H_2SO_4, and 120 min digestion.

METHOD

Reagents

(a) *Sodium hydroxide.*—Pellets, flakes, or solution with specific gravity 1.36 or higher, low nitrogen. Dissolve ca 450 g solid NaOH in water, cool, and dilute to 1 L.

(b) *Alundum.*—Boiling stones, 8–14 mesh (Arthur H. Thomas Co., Philadelphia, PA, No. 1590-D18).

(c) *Methyl red indicator.*—Dissolve 1 g methyl red (sodium salt) in 100 ml methanol.

(d) *Hydrochloric or sulfuric acid standard solution.*—0.5*N* or other desired concentration. Prepare as in 50.011 or 50.039 (7).

(e) *Sodium hydroxide standard solution.*—0.1*N* or other desired concentration. Prepare as in 50.032–50.034 (7).

(f) *Ammonium Dihydrogen Phosphate.*—NBS Standard Reference Material No. 194, certified 12.15±0.01% nitrogen (National Bureau of Standards, Washington, DC 20234).

Standardize each standard solution with primary standard (7), and check one against the other. Test reagents before use by blank determination with 1 g sugar, which ensures partial reduction of any nitrates present.

Ratio of salt to acid (weight/volume) should be about 1:1 at end of digestion for proper temperature control. Digestion may be incomplete at

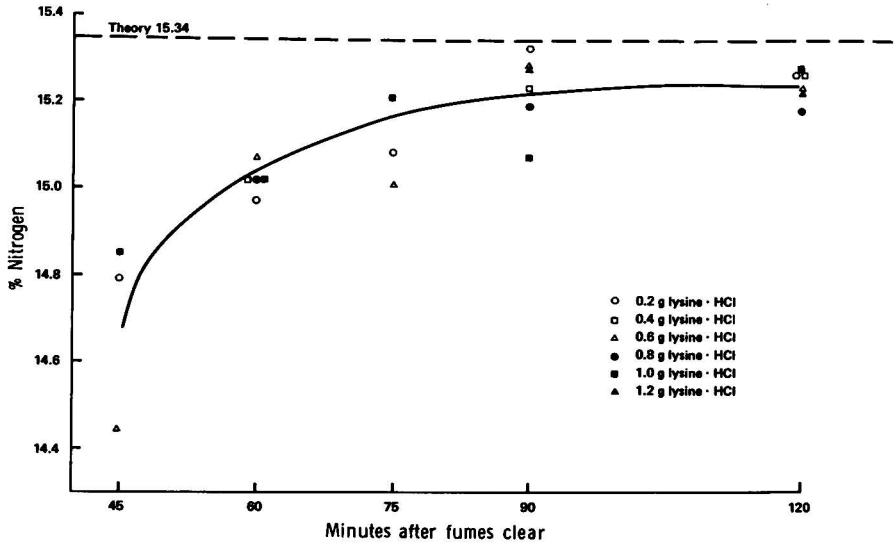


FIG. 2—Digestion time vs. nitrogen recovery (with varying weights of lysine . HCl).

lower ratio; nitrogen may be lost at higher ratio. Each 1 g fat consumes ca 10 ml H_2SO_4, each 1 g protein ca 5 ml, and each 1 g carbohydrate ca 4 ml during digestion.

Check performance by assaying NBS Standard Reference Material No. 194 and a high purity lysine.HCl.

Apparatus

(a) *For digestion.*—Use Kjeldahl flasks with total capacity ca 500–800 ml. Conduct digestion over heating device adjusted to bring 250 ml water at 25°C to rolling boil in ca 5 min. To test heaters, preheat 10 min if gas or 30 min if electric. Add 3–4 boiling chips to prevent superheating.

(b) *For distillation.*—Use 500–800 ml Kjeldahl flask, fitted with rubber stopper through which passes lower end of efficient scrubber bulb or trap (Corning No. 2020, 45 mm or a better bulb) to prevent mechanical carry-over of NaOH during distillation. Connect upper end of bulb tube to condenser tube by flexible low-sulfur tubing. Outlet of condenser tube should be ≤ 4 mm diameter. Trap outlet of condenser in such a way as to ensure complete absorption of NH_3 distilled over into acid in receiver.

Determination

Place 0.25–1 g sample in 500–800 ml Kjeldahl flask. Add 15 g K_2SO_4, 0.04 g anhydrous $CuSO_4$ or 0.06 g $CuSO_4.5H_2O$, 0.5–1 g alundum granules, and 20 ml H_2SO_4. If >1 g sample is desired, add additional 1.0 ml H_2SO_4 for each 0.1 g fat or 0.2 g of other organic matter.

Place flask on burners set at 5 min boil test. (Preheated burners reduce foaming with most samples. Cut back heat input if foam fills $>\frac{2}{3}$ of bulb of flask. Use variable heat input until this phase is past.) Heat at 5 min boil test until dense white fumes clear bulb of flask. Swirl flask gently and continue digestion 90 min more.

Cool, add ca 250 ml water (if solid NaOH is used, add ca 275 ml water), and cool below 25°C.

Position receiving flask containing standard acid and 3–4 drops of indicator solution so that tip of condenser is immersed in standard acid.

Add 2–3 drops of tributyl citrate to digestion flask to minimize foaming and ca 0.8 g alundum granules to minimize bumping. Tilt flask and add NaOH without agitation. *For each 10 ml H_2SO_4 used, add 15 g solid NaOH or enough solution to make contents strongly alkaline.* Immediately connect flask to distillation bulb on condenser. Swirl flask to mix contents rather well; then heat until all NH_3 has distilled (at least 150 ml distillate).

Remove receiver, wash tip of condenser, and titrate excess standard acid in distillate with standard NaOH solution. Correct for blank determination on reagents. Calculate % nitrogen. Crude protein, % = % nitrogen $\times$ 6.25 for grain and stock feeds. For wheat grains, crude protein, % = % nitrogen $\times$ 5.7.

Table 2. Ruggedness test variables

Variable	Designation	Values
Sample size, g	A, a	0.5, 1.0
Boil test, min	B, b	6, 5
K_2SO_4, g	C, c	14, 16
$CuSO_4$, mg	D, d	25, 60
H_2SO_4, ml	E, e	18, 22
Digestion time, min	F, f	82, 105
None	G, g	

Table 3. Results of ruggedness test

Condition	Lysine.HCl, % N	Diff., % N
1	15.33	Da −0.07
2	15.24	Db 0.02
3	15.21	Dc 0.01
4	15.27	Dd 0.03
5	15.31	De 0.01
6	15.34	Df 0.02
7	15.35	Dg 0.05
8	15.31	
Av.	15.30	
Std dev.	0.050	

Evaluation

A ruggedness test (11) was conducted with lysine.HCl. Such a test allows for an evaluation of 7 variables. These variables and the range of their values in the test are given in Table 2. The results of the ruggedness test are given in Table 3. The range of values of 15.21–15.35% nitrogen with a standard deviation of 0.050 vs. a theoretical value of 15.34% nitrogen is very good. This indicates that the method would perform well in an interlaboratory test.

The method was then compared with the AOAC official method, **2.049** (7). The comparison included 17 samples analyzed in duplicate on 3 different days. The amino acids and ammonium salt were dried 2 hr at 110°C and stored in a desiccator. Urea was dried 24 hr at room temperature in a desiccator over Drierite and kept there. Sample 1 was NBS Standard Reference Material 194, Ammonium Dihydrogen Phosphate. Sample 2 was ACS reagent grade urea.

Discussion

The analytical results are shown in Table 4. The apparent close agreement between the 2 methods was borne out by statistical evaluations which showed no difference at the 95% confidence level between the methods except for Sample 2. This difference of 0.05% nitrogen on 46% material would not cause any problems in our opinions. The Missouri copper method did have a slightly lower average standard deviation of 0.033 vs. 0.038. It also had a slightly higher average nitrogen value, 0.009% nitrogen per sample. Both methods checked with the

Table 4. Comparison of AOAC official (2.049) and Missouri copper catalyst methods

		Official method			Missouri Cu method			
Sample	Description	Av. % N	Std dev.	Rel. std dev.	Av. % N	Std dev.	Rel. std dev.	Diff., offic.−Cu
1	$NH_4H_2PO_4$[a]	12.09	0.033	0.27	12.14	0.025	0.21	−0.05
2	urea	46.55[c]	0.083	0.18	46.61[c]	0.090	0.19	−0.06
3	tryptophan	13.63	0.008	0.06	13.64	0.028	0.21	−0.01
4	lysine.HCl	15.29	0.015	0.10	15.30	0.026	0.17	−0.01
5	Orchard Leaves[b]	2.74	0.021	0.77	2.74	0.015	0.55	0.00
6	fish meal	9.93	0.037	0.37	9.93	0.026	0.26	0.00
7	meat and bone meal	7.89	0.043	0.54	7.87	0.031	0.39	0.02
8	blood meal	12.49	0.015	0.12	12.48	0.042	0.34	0.01
9	cottonseed meal	6.68	0.038	0.57	6.71	0.035	0.52	−0.03
10	soybean oil meal	7.14	0.026	0.36	7.13	0.019	0.27	0.01
11	corn grain	1.38	0.022	1.59	1.38	0.021	1.52	0.00
12	soybean grain	6.20	0.033	0.53	6.21	0.024	0.39	−0.01
13	wheat grain	2.23	0.013	0.58	2.23	0.009	0.40	0.00
14	grass	0.99	0.008	0.81	1.00	0.005	0.50	−0.01
15	mixed feed	6.72	0.086	1.28	6.69	0.043	0.64	0.03
16	starter ration	2.69	0.027	1.00	2.72	0.012	0.44	−0.03
17	concentrate	7.22	0.022	0.30	7.24	0.018	0.24	−0.02
Av.		9.52	0.038		9.53	0.033		−0.0094

[a] NBS Reference Material, No. 194, certified 12.15±0.01% N.
[b] NBS Reference Material, No. 1571, certified 2.76±0.05% N.
[c] Significantly different at 95% confidence level.

certified value on Sample 5. The Missouri copper method checked with the certified value (12.15±0.01% nitrogen) on Sample 1, whereas the official method gave a somewhat low value of 12.09% nitrogen. Results on Samples 2, 3, and 4 compare favorably with theory on the chemicals urea, tryptophan, and lysine.HCl. Both methods gave comparable results on Samples 6 through 17. They were selected to represent a cross-section of materials normally assayed using this methodology. The overall results of the comparison are that the Missouri copper method is equal to or slightly better in accuracy and precision than the AOAC official method 2.049 (7).

Conclusion

The method detailed above fulfills the goals of the authors in that mercury has been eliminated. Considering the low level of $CuSO_4$ used, real progress has been made in pollution reduction. This method was also part of a study covering 4 different methods for total protein nitrogen (12). The 2 methods in this paper were compared with the Kjel-Foss and the Missouri-Technicon with block digestor. All methods had excellent accuracy and precision.

The need for lysine.HCl with a certified nitrogen value is expressed. The lysine.HCl used in this study was satisfactory but a reliable analytical value rather than theoretical would be desirable. If enough analysts have an interest in this, the National Bureau of Standards might look favorably upon a request on this subject.

Contributed from the Missouri Agricultural Experiment Station. Journal series No. 7497. Approved by the Director. Received February 29, 1976.

Acknowledgments

The statistical data in Table 4 were provided by Gary Krause. He also provided helpful counsel in setting up the experimental design. Charles W. Gehrke, Larry L. Wall, Sr., Richard Antepenko, Carl Thompson, and numerous others of the staff have provided helpful guidance, comments, and analyses in contributing to the method and this paper.

References

(1) Dillon, P. L., Caldwell, M. J., & Gehrt, A. J. (1972) *JAOAC* **55**, 101–102
(2) Rexroad, P. R. (1972) *JAOAC* **55**, 707–708
(3) Odland, R. (1972) *JAOAC* **55**, 984–985
(4) Rexroad, P. R. (1973) *JAOAC* **56**, 862–863
(5) Johnson, F. J., Woodis, T. C., Jr., & Cummings, J. M., Jr. (1974) *JAOAC* **57**, 10–13
(6) Bates, R. W., Etheredge, M. P., & Quackenbush, F. W. (1955) *JAOAC* **38**, 56–61
(7) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC
(8) Bradstreet, R. B. (1965) *The Kjeldahl Method for Organic Nitrogen,* Academic Press, Inc., New York, NY
(9) Perrin, C. H. (1953) *Anal. Chem.* **25**, 968–971
(10) Shedd, O. M. (1927) *JAOAC* **10**, 507–520
(11) Youden, W. J. (1973) *Statistical Techniques for Collaborative Tests,* AOAC, Washington, DC
(12) Wall, L. L., Sr., Gehrke, C. W., Neuner, T. E., Cathey, R. D., & Rexroad, P. R. (1975) *JAOAC* **58**, 811–817

Collaborative Study of the Determination of Fat in Intermediate Moisture Pet Foods

GUSTAV O. KUHN
Quaker Oats Research Laboratories, 617 W Main St, Barrington, IL 60010

Four different semimoist pet food formulations representing a cross section of commercial products were studied collaboratively for fat content by 2 methods. Ten laboratories participated in the study. Direct petroleum ether extraction by AOAC method 7.045 yielded low and variable fat recovery. AOAC acid hydrolysis method 7.047 for fat in baked dog foods was satisfactory for semimoist pet foods. The method, with some editorial changes, was adopted as official first action for this type of pet food.

In early 1963, a new type of pet food, now categorically known as intermediate moisture, semimoist, or soft-moist pet food, was introduced into the commercial market. The rapid acceptance by the public of products containing meat that did not require refrigeration or have to be reconstituted with water resulted in a proliferation of similar formulations. At present, this type of pet food accounts for over 16% of all pet food sales in this country.

The unique characteristic of semimoist pet foods is the reduced water activity in relation to the total moisture content. This is achieved through the incorporation of substantial amounts of glycols (propylene glycol or glycerin), soluble carbohydrates (sugar, sorbitol, dextrins, corn sirup), and gelation agents such as starch or sodium caseinate. Acids such as phosphoric and sorbic acids are frequently added to inhibit microorganism growth.

For the regulatory chemist, the introduction of these intermediate moisture pet foods presented an analytical puzzle. These products are not feeds, and while some products can qualify as baked or expanded, many do not fulfill that definition. There is at present no official method for determining fat in this type of product.

This report is a followup of an earlier, unpublished collaborative study conducted in 1971, involving 6 different pet food products analyzed by AOAC method **7.050** (*Official Methods of Analysis* (1970) 11th Ed.). The same acid hydrolysis method (**7.047**, 12th Ed.) was used in the present study with analytical details more specifically defined as requested by collaborators in the first study.

Collaborative Study

Four representative pet food formulations chosen for this study were selected to bracket the extremes in composition that may be encountered in the analysis of commercial samples, and included products ranging from high to low sugar, high to low corn sirup, and high to low propylene glycol content. About 5 lb of each product was reduced to a uniform meal with a high-speed blender and then thoroughly hand-mixed before subsampling into 8 oz moisture-tight, screw-cap glass jars. Twice as many jars of products A and D were prepared for use as blind duplicates.

Each collaborator was mailed a set of 6 samples labeled A–F, an instruction sheet, a copy of the acid hydrolysis procedure, and reporting forms. The following samples were included in the 1975 study: A, high corn sirup, high propylene glycol, low sugar; B, no corn sirup, high propylene glycol, moderate sugar; C, no corn sirup, high propylene glycol, high sugar; D, no corn sirup, high propylene glycol, no sugar; E, duplicate of A; and F, duplicate of D.

Collaborators were asked to perform single determinations on 2 different days by the acid hydrolysis method and by method **7.045** (12th Ed.). Petroleum ether was recommended in place of ethyl ether for method **7.045**. This change was made for 2 reasons: (*1*) The large amount of water-soluble carbohydrate materials in semimoist products results in the extraction of significant quantities of non-fat substances by ethyl ether; and (*2*) the water pre-extraction step described in **7.045** is not applicable to all types of pet foods. Fat was lost during the water extraction on some samples, and the gelatinous character of several products rapidly seals the filter paper, making quantitative washing impossible. Two of the 6 samples sent to collaborators were blind duplicates to obtain additional information on sample uniformity and laboratory precision.

METHODS

I. Method **7.045** (12th Ed.), crude fat or ether extract, modified to use petroleum ether in place of ethyl ether.

II. Method **7.047** (12th Ed.), crude fat in baked or expanded pet food, edited to clarify details as shown below:

(To be used only on products all of which have been baked and/or expanded, and on intermediate moisture pet foods. Not applicable to canned, fresh, or frozen pet food. Such products should be dried at 70–110°, then ground, and drying completed by **7.003** or **7.007** followed by **7.045** or **7.046**. *Caution: See* **51.011, 51.054,** and **51.073**.)

Place ca 2 g, accurately weighed, ground, well mixed sample in Mojonnier fat-extn tube, add 2 ml alcohol to prevent lumping on addn of acid, and shake to moisten all particles. Add 10 ml HCl (25 + 11), mix well, and set tube 30–40 min in H_2O bath at 70–80°, shaking frequently. Cool to room temp. and add alcohol until liq. level is in constricted portion of Mojonnier tube.

Add 25 ml ether, stopper with glass, Neoprene, or good quality rubber stopper thoroly cleaned with alcohol, and shake vigorously 1 min. Carefully release pressure so that no solv. is lost. Wash adhering solv. and fat from stopper back into extn tube with few ml redistd pet ether (bp<60°). Add 25 ml redistd pet ether, stopper, and shake vigorously 1 min. Let stand until upper liq. is practically clear, or centrf. 20 min at ca 600 rpm. Pour as much of ether-fat soln as possible thru filter consisting of cotton pledget packed just firmly enough in funnel to let ether pass freely into 150 ml beaker contg several glass beads. Rinse lip with few ml pet ether. Re-ext liq. remaining in tube twice, each time with only 15 ml of each ether, shaking 1 min after addn of each ether. Pour clear ether soln thru filter into same beaker as before, and wash tip of tube, stopper, funnel, and end of funnel with few ml of mixt of 2 ethers (1+1). Evap. slowly on steam bath under gentle stream of air or N. Continue heating on steam bath 15 min after solv. has evapd; then cool to room temp.

Redissolve dried residue in four 10 ml portions of ether, filtering each portion thru small pledget of cotton into 100 ml beaker, contg few boiling chips, that has been predried 30 min at 100°, cooled to room temp. in desiccator, and weighed immediately. Use fifth 10 ml portion ether for rinsing cotton and funnel. Evap. ether on steam bath, dry 90 min at 100°, cool to room temp. in desiccator, and weigh immediately. Correct this wt by blank detn on reagents used.

Received September 12, 1975.

Results and Discussion

A comparison of mean fat values by the 2 methods (Tables 1 and 2) shows that the petroleum ether extraction method underestimates fat content by 9.4–18.5%. This observation is consistent with results obtained in the 1971 study in which 6 different pet food products were analyzed for fat by the same 2 methods. In that study (Tables 3 and 4), direct solvent extraction underestimated fat content by 7.1–65.5% as compared with the values obtained by acid hydrolysis. Table 5 shows the pet food types analyzed in the 1971 study.

As a result of suggestions made by collaborators after the 1971 study, procedure details as outlined in **7.047** (12th Ed.) were more clearly stated for collaborators. It is paradoxical that, while overall average coefficient of variation for all samples was slightly lower in the 1975 study compared with the 1971 study (5.56 vs. 6.18), there was a substantial variability in the 1975 study beyond the variability of open duplicates.

Under Youden's system of ranking laboratories (W. J. Youden (1973) *Statistical Techniques for Collaborative Tests*, AOAC, Washington, DC), 3 laboratories fell into a group separate from the others. Telephone interviews with the analysts revealed that these collaborators had changed significant method details in the acid hydrolysis procedure to conform more closely with modifications currently in use in their own laboratories. Some of these variations included using larger sample weights and larger acid volumes, digesting at a lower temperature than specified in the method, introducing a water-washing step, omitting the first drying and re-solution step, substituting separatory funnels for Mojonnier flasks, omitting filtration of the solvent extract, using filter paper instead of cotton, and drying the final fat residue only one-fourth as long as specified in the method. Since the purpose of the study was to evaluate a specific method, results from Collaborators 4, 7, and 10 were omitted from the statistical study on the basis of failure to follow the method.

The components of variance and their sources for data were calculated from analysis of variance of the 7 remaining laboratories: open duplicates, 0.20; blind duplicates, 0.33; and laboratories, 0.47. These figures show that while repeatability of ordinary open duplicates is ac-

Table 1. Collaborative results for % fat in semimoist pet foods, petroleum ether extraction—1975 study

Coll.	Day	Sample A	B	C	D	E	F
1	1	10.33	7.04	9.54	9.86	10.11	10.03
	2	10.28	7.17	9.47	9.93	10.21	10.05
2	1	10.20	6.50	9.30	9.70	10.40	9.90
	2	9.70	6.90	8.90	9.80	9.80	9.60
3	1	10.10	7.19	9.67	8.07[a]	9.66	9.85
	2	9.93	7.23	9.69	8.13[a]	9.95	9.87
4	1	10.72	7.90	9.97	9.97	10.86	—
	2	10.66	7.71	9.96	10.06	10.37	10.02
5	1	10.00	7.30	9.30	9.50	10.00	9.60
	2	9.50	7.30	9.10	9.60	10.20	9.60
6	1	10.10	7.50	9.80	10.10	10.20	10.40
	2	10.00	7.60	10.00	10.30	10.30	10.10
7	1	10.75	7.43	10.00	10.50	10.60	9.17[a]
	2	10.71	8.04	10.30	10.70	10.70	10.62[a]
8	1	10.29	7.28	9.53	9.73	10.09	9.68
	2	10.20	7.22	9.59	9.59	10.54	9.62
9	1	10.33	7.25	9.63	7.85[a]	10.03	9.68
	2	10.46	7.32	9.82	9.77	10.08	9.69
10	1	8.85[a]	7.79	9.85	9.85	10.13	—
	2	10.70	7.26	9.27	12.17[a]	10.62	10.05
N		19	20	20	16	20	17
Mean		10.26	7.35	9.63	9.94	10.24	9.82
Var.		0.1183	0.1156	0.1129	0.1024	0.0900	0.0750
Std dev.		0.344	0.340	0.337	0.320	0.300	0.274
Coeff. of var., %		3.35	4.62	3.50	3.22	2.93	2.79

[a] Varies by more than 2 standard deviations. Not included in mean.

ceptable, there is at least as much additional variability within laboratories, and a similar amount from laboratory to laboratory. A small portion of this additional variability determined on blind duplicates may be assignable to simple variability in the sample.

Two of the collaborators (5 and 9) exhibited a definite bias toward low results relative to the other 5 collaborators (5% significance level). This is based on a Duncan multiple-range test of the laboratory means. These laboratories were indistinguishable from each other at the 5% significance level but a significant gap was found between them and the remaining means. By the same test, Collaborator 2 showed a tendency to high results for all products by acid hydrolysis. Collaborators 3 and 9 had a relatively high random variability based on an analysis of deviations from the diagonal on a Youden 2-sample plot.

The 1975 study again confirms that some familiarity with the method in routine use generally yields more reproducible values. The gross standard deviation for a single assay, unduplicated, at an unspecified laboratory is 0.61, which is equivalent to a coefficient of variation of 5.6% at the observed average fat level of 10.84%. This is a slight improvement compared with a gross standard deviation of 0.68 and a coefficient of variation of 6.1% on a mean fat content of 11.07% found in the 1971 study. Gross standard deviation is the standard deviation to be expected when a sample is analyzed once at an unspecified laboratory taken from a population of laboratories of which the present 7 are considered to be a random sample. It is calculated by adding together the appropriate components of variance and taking the square root of the resulting sum.

Several collaborators expressed the opinion that redissolving the dried fat at the end of the method was unnecessary. The Associate Referee agrees that this is true for some pet foods; however, other commercial samples cause a significant carry-over of non-fat materials. This was clearly demonstrated in the 1971 study, and it is not recommended that this operation be eliminated unless the laboratory has determined

Table 2. Collaborative results for % fat in semimoist pet foods, acid hydrolysis—1975 study

Coll.	Day	Sample A	B	C	D	E	F
1	1	11.61	8.94	10.60	11.31	11.36	11.20
	2	11.72	8.98	10.71	11.26	11.74	11.24
2	1	11.70	9.60	11.50	11.70	11.70	11.90
	2	12.30	9.90	11.30	11.90	12.40	11.70
3	1	11.45	8.77	10.67	11.17	12.15	12.32
	2	11.47	8.94	10.67	11.14	12.32	12.37
4[a]	1	9.68	7.62	9.58	9.98	8.79	9.77
	2	11.15	8.41	10.26	10.63	—	10.47
5	1	11.20	8.40	10.00	10.00	10.80	10.30
	2	11.00	7.80	10.00	10.50	10.90	10.20
6	1	11.70	8.80	10.80	11.10	11.80	11.20
	2	11.60	8.90	10.90	11.40	12.00	11.30
7[a]	1	10.71	8.71	9.97	9.84	10.80	10.20
	2	10.96	8.41	9.36	9.12	10.64	9.46
8	1	11.55	9.21	10.62	11.63	11.69	11.30
	2	12.08	9.21	11.08	11.62	11.85	11.48
9	1	11.52	9.05	10.68	9.31	9.96	—
	2	11.47	9.80	10.44	9.53	9.74	10.51
10[a]	1	11.05	8.15	8.60	9.78	10.08	9.93
	2	10.72	8.06	8.61	10.01	9.74	9.70
N		14	14	14	14	13	13
Mean		11.60	9.02	10.71	10.97	11.46	11.31
Var.		0.1031	0.2927	0.1737	0.6706	0.6781	0.4662
Std dev.		0.321	0.541	0.411	0.819	0.823	0.683
Coeff. of var., %		2.77	6.00	3.84	7.46	7.18	6.04

[a] Omitted due to departure from procedure.

that the particular samples being analyzed do not cause carry-over errors. One collaborator proposed a mild alkaline water wash of the mixed ether extract prior to drying. This suggestion may have merit, provided the free fatty acid content of the samples is always low.

Conclusions and Recommendations

The results from 2 collaborative studies show that Method **7.047** for baked or expanded pet foods can be applied to determining fat content of intermediate moisture (semimoist) pet foods. The direct petroleum ether extraction gives low and widely varying recoveries of fat with these products.

It is recommended—

(*1*) That **7.047** be designated as official first action for intermediate moisture (semimoist or soft-moist) pet foods.

(*2*) That the title of **7.047** be changed to: "In Baked or Expanded Pet Food and Intermediate Moisture (Semimoist or Soft-Moist) Pet Foods."

(*3*) That the text of the present method (**7.047**) be edited to incorporate the changes included in this study. The revised version is shown under *Method*.

Acknowledgments

The Associate Referee wishes to express appreciation to the following collaborators for their participation in this study:

Robert Boswell, Armour Food Co., Chicago, IL

E. Daskalakis, Rosner Hixon Labs, Chicago, IL

Jim Grove, General Foods Corp., Battle Creek, MI

Larry Hambleton, Purdue University, West Lafayette, IN

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee A and were adopted by the Association. *See* (1976) *JAOAC* **59**, 378.

This report of the Associate Referee was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Table 3. Collaborative results for % fat in semimoist pet foods, petroleum ether extraction—1971 study

		Sample					
Coll.	Day	A	B	C	D	E	F
1	1	10.25	7.05	4.03	9.68	9.50	9.10
	2	10.75	7.30	4.25	10.20	9.91	8.65
2	1	10.40	7.50	5.07	9.83	9.34	8.86
	2	10.40	7.48	5.06	9.58	9.23	8.87
3	1	10.22	7.80	4.86	9.89	9.79	8.72
	2	10.24	7.67	4.83	9.73	9.28	9.00
4	1	10.29	7.88	2.62	10.04	9.66	9.18
	2	9.93	7.29	2.79	9.99	9.48	9.05
5	1	10.47	7.95	4.72	9.87	10.60[a]	9.47
	2	10.35	7.81	4.58	10.04	9.56	9.35
6	1	11.43[a]	10.80[a]	5.29	10.91[a]	9.76	10.51
	2	11.08	9.82	5.24	11.29[a]	9.90	10.86[a]
7	1	10.40	7.38	2.81	9.78	9.22	8.76
	2	9.80	6.34	2.78	9.68	9.54	8.89
8	1	10.46	7.91	5.09	10.00	9.98	9.83
	2	10.65	7.99	4.97	10.09	9.95	9.69
N		15	15	16	14	15	15
Mean		10.38	7.68	4.31	9.88	9.61	9.20
Var.		0.0882	0.5013	0.9158	0.0306	0.0666	0.2381
Std dev.		0.297	0.708	0.957	0.175	0.258	0.488
Coeff. of var., %		2.86	9.22	22.20	1.77	2.68	5.30

[a] Varies by more than 2 standard deviations. Not included in mean.

Table 4. Collaborative results for % fat in semimoist pet foods, acid hydrolysis—1971 study

		Sample					
Coll.	Day	A	B	C	D	E	F
1	1	12.64	10.27	12.41	11.17	11.36	10.40
	2	12.78	10.19	12.50	11.06	11.38	10.39
2	1	12.80	13.90[a]	12.20	10.90	12.00	11.20
	2	12.30	13.10[a]	12.70	11.10	11.40	11.00
3	1	11.60	8.79	11.36	9.83	10.97	10.54
	2	11.94	8.63	11.40	10.09	10.47	9.75
4	1	13.28	9.17	12.55	10.26	11.14	9.88
	2	13.35	9.46	13.18	10.89	11.75	10.18
5	1	12.08	8.54	12.26	10.01	10.96	9.90
	2	11.82	8.51	12.04	10.13	10.85	9.84
6	1	13.31	10.32	13.40	11.37	12.82	11.66
	2	13.09	10.00	13.73	11.58	12.72	11.99[a]
7	1	11.70	9.14	12.20	10.40	10.40	9.80
	2	11.40	8.80	11.80	9.63	10.40	9.70
8	1	13.90	10.90	13.40	11.40	12.40	11.10
	2	13.40	9.80	12.80	10.50	12.00	10.65
N		16	14	16	16	16	15
Mean		12.59	9.47	12.50	10.64	11.44	10.40
Var.		0.5520	0.5655	0.4489	0.3528	0.5746	0.3540
Std dev.		0.743	0.752	0.670	0.594	0.758	0.595
Coeff. of var., %		5.90	7.94	5.36	5.58	6.63	5.72

[a] Varies by more than 2 standard deviations. Not included in mean.

Table 5. Identification of pet food product types tested in 1971 collaborative study

Sample	Product
A	dog food, beef by-product base
B	dog food, chicken by-product base
C	dog food, synthetic meat analog type
D	dog food, beef and chicken by-product base
E	dog food, beef by-products with cheese
F	cat food, chicken by-product, no sugar

Frances Mangine and Vickie Olson, Quaker Oats Co., Barrington, IL

Robert Moffett, Carnation Co., Van Nuys, CA

Cara Jo Peck, Quaker Oats Co., Rockford, IL

Earl Richter, WARF Institute, Inc., Madison, WI

Virginia Thorpe, Michigan State Department of Agriculture, East Lansing, MI

DECOMPOSITION IN FOODS (CHEMICAL INDEXES)

Rapid Thin Layer Chromatographic Method for the Detection of Histamine in Fish Products

DAVID E. SCHUTZ, GEORGE W. CHANG, and LEONARD F. BJELDANES
Department of Nutritional Sciences, University of California, Berkeley, CA 94720

A rapid, convenient thin layer chromatographic (TLC) method for detecting histamine in fish samples is described. Samples of press juice or fish flesh are applied directly to TLC plates. The plates are developed with acetone-ammonium hydroxide (95+5) and the spots are visualized with ninhydrin or Pauly's reagent. Chromatographic separation of histamine from other fish components is readily achieved by this method.

The presence of histamine in scombroid fish (tuna, mackerel, bonito), herring, and dolphin (mahi-mahi) is associated with bacterial spoilage and, occasionally, human illness (1). The detection of high levels of histamine in commercial fish products has resulted in regulatory action and the subsequent recall of several lots (2). The analytical methods now in use for detecting histamine in fish products are time consuming and/or costly (3–5). These methods cannot be used for routine screening of large numbers of samples under production-line conditions. We have developed a simple, inexpensive thin layer chromatographic (TLC) method for detecting histamine in fish and other products. The method is semiquantitative, yet convenient and sensitive enough to be used in the routine analysis of large numbers of samples.

Experimental

Apparatus and Reagents

(a) *Thin layer chromatographic plates.*—Poly(polyethylene terephthalate) sheets, pre-coated with 0.100 mm silica gel (Eastman Chromagram 13181, Eastman Kodak Co., Rochester, NY 14650). Cut to 8 cm × width to accommodate number of samples to be examined.

(b) *Pipets.*—Disposable, capillary; 5 μl capacity (Dade Diagnostics, Inc., Miami, FL 33152).

(c) *Developing solvent.*—ACS grade, or equivalent. Acetone-ammonium hydroxide (28 wt% ammonia) (95+5).

(d) *Histamine standard solution.*—1 mg/ml. Dissolve 165.8 mg histamine dihydrochloride (Sigma Chemical Co., St. Louis, MO 63178) in 100.0 ml water.

(e) *Pauly's spray reagent.*—Immediately before use, combine Pauly's reagent (6) and aqueous 12% (w/v) Na_2CO_3 (2+5, v/v). Lightly spray solution onto plates.

(f) *Ninhydrin.*—Aerosol (1-4496, J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(g) *Histamine standard fish mixture.*—165.8 mg histamine dihydrochloride (100 mg% histamine)/100 g fish flesh.

(h) *Spoiled tuna samples.*—6.5 oz cans from lots D417 and D419 which have been implicated in outbreaks of human poisoning (2).

Preparation of Samples

(a) *Tuna packed in oil.*—Let press juice run from opened can, pressing lid into fish with moderate pressure. Separate aqueous and oil phases using a separatory funnel and let particles settle out of aqueous phase until it is clear.

(b) *Tuna packed in brine.*—Let press juice run from opened can as above. Let settle until clear.

(c) *Fish flesh.*—Flesh samples should include no skin and may come from any suspected areas of muscle. Samples should be spherical chunks ca 0.5 cm in diameter.

Thin Layer Chromatography

Spot 5 μl aqueous press juice, or press piece of flesh to sheet with spatula until area underneath is moist. Also place aliquot of standard histamine solution on plate. Fish flesh and aqueous press juice should be placed on plate ≥2 and 1.5 cm, respectively, from adjacent spots. Air-dry TLC plate and then develop with acetone-NH_4OH (95+5) until solvent front has advanced ≥6 cm past origin. This elution requires approximately 10–12 min. Air-dry and spray plate with desired reagent.

This work was supported by a grant from the NOAA office of Sea Grant, United States Department of Commerce (UCSG-5, R/F-22).

Results and Discussion

Histamine elutes with an R_f of 0.54 in the acetone-ammonium hydroxide system described, and produces purple spots when sprayed with ninhydrin and orange spots with Pauly's reagent. We prefer Pauly's reagent because the color develops faster and is more permanent. Histamine levels as low as 2.5 mg% in fish samples and standard solutions are readily detected. Other fish components visible with these spray reagents, including histidine, have very low mobility with this eluant (R_f <0.1) and are clearly separated from histamine.

The following evidence indicates that we detect histamine and only histamine in a naturally spoiled sample of tuna: spoiled fish samples known to contain high histamine levels contain a substance which co-elutes with histamine in the acetone-ammonium hydroxide solution and in the following solvent systems: acetone-chloroform-water-ammonium hydroxide (30+5+4+0.2); pyridine-ammonium hydroxide-water (35+35+30); isobutyric acid-water (4+1); and methanol-ammonium hydroxide (95+5). Furthermore, in a series of 2-dimensional TLC experiments using these latter solvents as eluants for one dimension and acetone-ammonium hydroxide for the other dimension, the substance in spoiled fish which co-elutes with histamine gives uniformly homogeneous, single spots.

The elution properties of the acetone-ammonium hydroxide system change slowly with time with a resultant decrease in the R_f of histamine. Thus, the R_f of histamine decreases from 0.54 to 0.33 as the eluant is allowed to stand at room temperature for 2 weeks. This change appears to be due to volatilization of ammonia, since addition of ammonium hydroxide to the aged solution restores its original elution properties. We suggest that fresh acetone-ammonium hydroxide solution be prepared at 3-day intervals.

Histamine levels which may be of regulatory concern in fish (i.e., 20 mg%) are determined rapidly and at low cost by this analytical technique. It is suggested that the method be used for routine screening for determining which fish samples may contain unusual histamine levels. Suspect lots may then be assayed by the more time-consuming, established, quantitative methods.

References

(1) Edmunds, W. J., & Eitenmiller, R. R. (1975) *J. Food Sci.* **40**, 516–519

(2) Center for Disease Control (1973) *Morbidity and Mortality Weekly Report* **22**, 69

(3) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, sec. **18.060**

(4) Code, F. C., & McIntire, F. C. (1954) in *Methods of Biochemical Analysis*, Vol. 3, D. Glick (Ed.), Interscience Publishers, New York, NY, pp. 49–95

(5) Shore, P. A. (1971) in *Methods of Biochemical Analysis*, Suppl. Vol., D. Glick (Ed.), Interscience Publishers, New York, NY, pp. 89–97

(6) Hais, I. M., & Macek, K. (1963) in *Paper Chromatography, a Comprehensive Treatise*, Academic Press, New York, NY, p. 786

Received May 3, 1976.

METALS AND OTHER ELEMENTS

A Simplified Method for the Gas-Liquid Chromatographic Determination of Methyl Mercury in Fish and Shellfish

JAMES O. WATTS, KENNETH W. BOYER, ANTHONY CORTEZ,* and EDGAR R. ELKINS, JR.*

Division of Chemistry and Physics, Food and Drug Administration, Washington, DC 20204

** National Canners Association, 1133 20th St, NW, Washington, DC 20036*

A simple acetone wash of the fish sample which removes lipids and other organic materials replaces the cysteine cleanup specified in other methods. Methyl mercury is freed by hydrochloric acid, extracted into benzene, and determined with a gas-liquid chromatograph equipped with an electron capture detector. The method is quantitative for methyl mercury levels as low as 0.10 ppm in fish and shellfish. Ethyl mercury chloride may be used as an internal standard to detect unsuspected error or instrumental parameter variation.

Mercury is widely distributed in the environment, both from natural geological activity and from industrial pollution. Conversion of inorganic mercury to highly toxic methyl mercury by aquatic organisms is now well known and well understood (1, 2). Because of the rapid uptake of methyl mercury and because of its long biological half-life, the major form of mercury contamination found in fish muscle is methyl mercury (3). In general, ethyl mercury is not found in fish and shellfish. In one unusual case involving direct contamination of a river with ethyl mercury and inorganic mercury, however, accumulation of an ethyl mercury level higher than that for methyl mercury in the same fish has been reported (4). Schafer *et al.* (5) reported one sample of trout suspected of containing ethyl mercury. Methods of direct analysis for organic mercury in fish and shellfish in general use are the Westöö methods (6, 7), the Uthe *et al.* method (8), and the Magos method (9), or modifications of the above methods.

Essentially the Westöö methods (6, 7) consist of adding hydrochloric acid to a homogenized fish sample to convert methyl mercury bound to the protein to methyl mercury chloride. Methyl mercury chloride is extracted into benzene and then into aqueous cysteine solution to eliminate interfering organic material; the extract is acidified with concentrated hydrochloric acid to liberate methyl mercury from the cysteine complex again as methyl mercury chloride, which is then re-extracted into benzene for gas-liquid chromatographic (GLC) determination. In the Westöö methods, partitioning of methyl mercury in each step is not quantitative and a correction factor based on extraction partitioning coefficients for a model system must be used to calculate the concentration of methyl mercury originally present in the sample.

The method of Uthe and coworkers (8) consists of extracting methyl mercury from a homogenized sample into toluene as methyl mercury bromide, partitioning the bromide into aqueous ethanol as a thiosulfate complex, and re-extracting into benzene as methyl mercury iodide for determination by GLC.

The Magos method (9) consists of alkaline digestion of a homogenized sample followed by atomic absorption spectrometric determination of either total mercury or inorganic mercury. Total mercury is released as mercury metal by adding a stannous chloride-cadmium chloride complex reagent to the sample. Inorganic mercury is released as mercury metal by adding stannous chloride only to the sample. Organic mercury is determined by difference. This method does not differentiate methyl mercury from other forms of organic mercury.

Because the above methods either are tedious or have other objectional features and because a high percentage of the mercury in fish and shellfish usually exists as methyl mercury, a simple, more rapid method (10), which determines total mercury only, was chosen to support the Food and Drug Administration's 0.5 ppm guideline for mercury in fish and shellfish (11).

A suitable method for the direct analysis for methyl mercury, which is rapid, simple, and capable of accurately measuring methyl mercury

at levels as low as 0.10 ppm in fish and shellfish, is desirable. The method described here was developed with these needs in mind.

METHOD

Principle

Lipids and other organic materials are removed by acetone followed by benzene extraction. Protein-bound methyl mercury and ethyl mercury are released with HCl, extracted into benzene, and determined by GLC.

Reagents

Use distilled deionized water throughout. Clean all glassware with 25% NaOH initially; then rinse successively with water, HCl (1+1), water, and acetone.

(a) *Benzene.*—Distilled-in-glass (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442). After concentration of 50 ml to 10 ml in 250 ml Kuderna-Danish concentrator fitted with 10 ml concentrator tube, 10 μl injection into gas chromatograph under same conditions as for samples should show no peaks after benzene solvent front.

(b) *Hydrochloric acid solution.*—6*N*. Dilute concentrated HCl (ACS grade) with water (1+1). Extract solution 5 times with ¼ its volume of benzene.

(c) *Methyl and ethyl mercury chloride standard solutions.*—(*1*) *Stock solutions.*—1000 μg of each component/ml benzene. Weigh 100 mg of each compound (K&K Laboratories, Inc., 121 Express St, Plainview, NY 11803) into separate 100 ml volumetric flasks and dilute to volume with benzene. (*2*) *Intermediate solutions.*—50 μg/ml. Dilute 10 ml of each stock solution to 200 ml with benzene. (*3*) *Spiking solutions.*—2.5 μg/ml. Dilute 10 ml of each intermediate solution to 200 ml with benzene. (*4*) *Working solutions.*—0.1 μg/ml. Dilute 1 ml of each spiking solution to 25 ml with benzene. Standard solutions containing >1 μg/ml benzene are very stable. Prepare more dilute solutions at least monthly.

(d) *95% Argon-5% methane gas mixture.*—(Air Products, Specialty Gases Department, PO Box 538, Allentown, PA 18105).

(e) *Sodium sulfate.*—Anhydrous, ACS reagent grade. Heat overnight in 600°C muffle furnace, let cool, and store in capped brown bottle. Line cap with aluminum foil to prevent organic contamination from cap.

Apparatus

(a) *Centrifuge.*—Model UV, with No. 240 or 241 head and No. 341 container (International Equipment Co., Needham Heights, MA 02194).

(b) *Centrifuge bottles.*—50 ml, No. 16 ⅎ stopper (No. K-411650, Kontes Glass Co., Vineland, NJ 08360), or equivalent.

(c) *Gas chromatograph.* — Hewlett-Packard Model 5710A equipped with linear electron capture detector and 4′ × 4 mm id glass column; column packing: 15% diethylene glycol succinate (DEGS) polyester on 80–100 mesh Gas-Chrom P (Applied Science Laboratories, State College, PA 16801). Pack column with DEGS to within 1″ of heated injection port; pack remainder with silanized Pyrex glass wool; change glass wool frequently to ensure good reproducibility of GLC peaks. Condition column 2 hr at 100°C with carrier gas flow rate of 5–10 ml/min followed by 48 hr at 200°C with no carrier gas flow; finally condition 4 hr at 225°C with carrier gas flow rate of 75 ml/min.[1] Operating conditions: temperatures (°C)—column 160, detector 350, injection port 250; attenuation 8×; recorder chart speed 0.5″/min; 95% argon-5% methane carrier gas mixture at 90 ml/min. Under these conditions, methyl mercury chloride and ethyl mercury chloride elute in ca 3 and 5½ min, respectively, after sample injection.

(d) *Filter.*—15 ml porous glass, ASTM type, medium porosity (Pyrex, No. K-955000, Kontes Glass Co.), or equivalent. If several samples are to be analyzed, use of following is convenient: Chromaflex column (Size 233, No. 420540, Kontes Glass Co.), 300 mm × 25 mm od, with coarse porosity fritted disk and Teflon stopcock. Modify by cutting column 3″ above fritted disk. Since each column flow rate is controlled with a stopcock, a single vacuum source with appropriate manifold system may be used. In this laboratory the manifold system consisted of glass "T" tubes connected to each vacuum flask.

(e) *Filter flask.*—250 ml with one vacuum arm (No. K-617500, Kontes Glass Co.), or equivalent.

(f) *Evaporative concentrator.*—Kuderna-Danish flask 250 ml with 19/22 ⅎ joint for concentrator tube connection (No. K-570001, Kontes Glass Co.); Snyder distillation column 24/40 ⅎ joints, 3 sections (No. 6575-02, Ace Glass Inc., Vineland, NJ 08360) (modify for benzene evaporation by cutting off third bubble section at glass seal joint between second and third bubble sections); and 10 ml concentrator tube, size 1025, with 19/22 ⅎ joint, and 19/22 ⅎ stopper (No. K-570050, Kontes Glass Co.), or equivalent.

(g) *Pipet.*—50 ml. Cut delivery tip flat 1″ from tip end.

[1] Although a conditioning temperature of 225°C is 25°C above the recommended maximum temperature for DEGS, we did not find any detrimental effects after a 4 hr conditioning at this temperature.

(h) *Pipet filler bulb.*—Valve-type (No. 13-681-50, Fisher Scientific Co., Pittsburgh, PA 15219), or equivalent.

Preparation of Sample

(a) *Canned, fresh, or frozen fish.*—If frozen, thaw first. Remove nonedible parts. Weigh fish sample and transfer to blender (5 g sample is required for each replicate). Add weight of distilled water equal to exactly 10% of weight of fish and blend mixture at medium speed 2–3 min until homogeneous slurry is obtained. Transfer slurry to glass or plastic jar, cap tightly, and store in refrigerator until needed. If homogenized sample is frozen, thaw, and rehomogenize before analyzing. (*Note:* 5.5 g prepared sample is equivalent to 5.0 g original sample.)

(b) *Oysters, clams, and other hard-shelled shellfish.*—Remove shell. Separate edible portion and wash with distilled water. Drain to remove excess water, weigh, transfer edible portion to blender, and proceed as in (a).

(c) *Shrimp and lobster.*—Peel, devein, and rinse edible portion with distilled water. Drain, weigh, transfer edible portion to blender, and proceed as in (a).

Isolation of Methyl Mercury Chloride

Transfer 5.5 g sample prepared as above into porous glass filter funnel. Place glass filter into one-hole No. 6 rubber stopper mounted in 250 ml filter flask with no vacuum applied. Add 10 ml acetone and stir sample with small glass rod. Apply enough vacuum to pull acetone from filter funnel at fast drip rate and let most of acetone drain into flask. Add 15 ml additional acetone, stir, and again vacuum-drain. Add continuously, with vacuum applied and with stirring, additional 100 ml acetone. When acetone is almost drained from sample, add 25 ml benzene continuously. Drain benzene and let sample dry 5 min with vacuum applied. (*Note:* The analysis may be stopped overnight either after the sample is air-dried in the porous glass filter for 5 min or before the benzene solution is concentrated in the Kuderna-Danish apparatus. Otherwise the analysis should not be stopped until the final 25 ml solution for GLC determination is obtained.) Scrape sample free with small, flat-edge spatula and quantitatively transfer sample powder to 50 ml centrifuge tube, using small powder funnel. Wash glass filter and spatula with total of 20 ml 6*N* HCl and add washings to 50 ml centrifuge tube. In same manner, wash glass filter and spatula with 5 ml isopropanol, followed by 25 ml benzene, and add to centrifuge tube. (The centrifuge tube specified actually holds 60 ml.) Stopper tube, shake vigorously 2 min, and centrifuge 15 min at 2000 rpm. If gel forms, gently reshake and recentrifuge. Clamp 50 ml modified pipet to laboratory support stand above centrifuge tube with pipet tip extending into centrifuge tube to ¼″ above benzene-aqueous interface. Without disturbing interface draw benzene layer into 50 ml pipet, using valve-type pipet bulb. Rinse sides of tube with 2–3 ml benzene, using glass medicine dropper. Continue to withdraw benzene rinse layer into 50 ml pipet as before.

In same manner, repeat with another benzene rinse of centrifuge tube walls. Transfer benzene in 50 ml pipet into Kuderna-Danish apparatus and rinse pipet with benzene. Repeat above extraction and centrifuge 2 more times with 25 ml benzene each time. (If gel formation occurs in second or third 25 ml benzene extraction of methyl mercury chloride from acidified sample, add additional 1 ml isopropanol; then reshake and recentrifuge prior to withdrawal of benzene layer.) Use 1 min extraction-shaking times and combine extract in Kuderna-Danish apparatus. Wet Snyder column bubble chambers with benzene and evaporate sample solution on steam bath to ca 8 ml (do *not* evaporate to <8 ml as ethyl and methyl mercury chloride losses may occur). Let apparatus cool; then disconnect concentrator tube and quantitatively transfer benzene to 25 ml glass-stoppered graduated cylinder. Rinse concentrator tube with small amounts of benzene and add to graduated cylinder. Dilute to 25 ml with benzene and then add 5 g anhydrous Na_2SO_4 to keep benzene dry. Reserve for GLC determination (25 ml represents 5 g original sample).

Gas-Liquid Chromatography

Inject measured volumes of working solutions (5 µl is recommended) separately into gas chromatograph, using GLC parameters described under *Apparatus*. Repeat injection until reproducible (constant within 3%) peak heights are obtained for both methyl and ethyl mercury chloride; 0.5 ng methyl mercury chloride should give ½ full-scale deflection. Inject sample solution. Periodically check retention times and response by injecting standard solution. If GLC system response is not linear, use standard solution that closely approximates height of sample peak. Calculate concentrations in original sample by comparing peak heights for sample and standard as follows:

$$\text{Methyl (or ethyl) mercury chloride, ppm} = (R/R') \times (C'/C) \times (V'/V)$$

where R and R' = peak height responses (mm) for sample and standard, respectively; C and C' = concentrations of sample in sample solution (g

original sample/ml sample solution) and standard (μg/ml), respectively; V and $V' =$ volume (μl) of sample and standard solutions injected, respectively.

To calculate results as ppm elemental mercury, multiply methyl mercury chloride results by 0.80 (molar factor for mercury in methyl mercury chloride). For ethyl mercury chloride, use factor of 0.76.

Recovery Studies

The recovery of methyl and ethyl mercury chloride can be determined by using the procedure described above with the following modification: After washing weighed sample with total of 25 ml acetone (to remove water which is immiscible with standards in benzene), let sample settle on porous glass frit without going dry. With vacuum off, add 1 ml standard containing 2.5 μg of each compound/ml (0.50 ppm each for 5.0 original sample) directly on upper surface of sample. Using small glass rod, gently stir upper portion of sample without disturbing portion directly on surface of glass frit. Wash rod with acetone into sample. Apply vacuum and continue to wash sample until total of 125 ml acetone and 25 ml benzene pass through filter. Let sample dry 5 min with vacuum applied. Continue analysis as described above for unspiked samples. Compare results with spiked samples to determine recoveries.

Results and Discussion

Samples of tuna, cod, mackerel, shrimp, lobster, and oysters were analyzed for both methyl and ethyl mercury, using the procedure described above. In addition, representative homogenized samples were spiked with methyl and ethyl mercury chloride, each at the 0.5 and 0.1 ppm levels, and carried through the analytical procedure to determine typical recoveries.

Table 1 lists values of methyl mercury found in unspiked samples, as well as recovery data for added methyl and ethyl mercury chloride. As Table 1 shows, the method was used to analyze samples with native mercury levels from 0.85 ppm (bluefin tuna) to $<$0.01 ppm (oysters). No ethyl mercury was detected in any of the samples analyzed; the detection limit is approximately 0.01 ppm. In one sample of oysters a large interference peak made analysis for ethyl mercury impossible. Analysis of the same oyster sample with the Munns method (12) for total mercury showed that the sample contained 0.01 ppm mercury, indicating that the interfering peak having a slightly shorter retention time than ethyl mercury chloride was not the result of a mercury-containing compound.

The skipjack and albacore tuna samples were previously analyzed in an interlaboratory study by 15 laboratories (unpublished data, 1972). In these analyses the skipjack tuna was found to contain 0.086$\pm$0.022 ppm methyl mercury (as mercury) compared to 0.093$\pm$0.005 ppm in this study. Likewise the albacore tuna was found to contain 0.44$\pm$0.09 ppm methyl mercury (as mercury) compared to 0.46$\pm$0.013 ppm in this study.

The recovery data in Table 1 range from a low of 94% to a high of 108% with an average recovery for all samples of 99.1$\pm$2.3% for methyl mercury chloride and 100.3$\pm$3.4% for ethyl mercury chloride at the 0.5 ppm spiking level and 100.1$\pm$4.2% and 97.9$\pm$2.4%, respectively, at the 0.1 ppm spiking level. No recoveries were determined by direct spiking of samples at levels above 0.5 ppm. However, one sample with a 0.76 ppm native methyl mercury level and spiked with 0.50 ppm methyl mercury chloride (total mercury, 1.16 ppm as mercury) showed quantitative recovery. We believe that methyl mercury levels from 1.0 to 5.0 ppm could be determined by diluting the final benzene extract used for the determination.

Figure 1A shows the chromatogram of an unspiked tuna sample found to contain 0.65 ppm methyl mercury chloride. The interfering background is very low and no ethyl mercury chloride peak is present. Figure 1B shows a chromatogram of a standard solution of both compounds in benzene at the 0.5 ppm level. Figure 2A is a chromatogram of the background for unspiked lobster with several unknown peaks preceding the peaks for methyl and ethyl mercury chloride. While a small peak was observed at the retention time of methyl mercury chloride, no peak was visible which corresponded to ethyl mercury chloride. When the lobster sample is spiked with 0.10 ppm of each compound, the same background is observed (Fig. 2B) with

The recommendation of the Associate Referee was approved by the General Referee and Subcommittee E and was accepted by the Association. Their reports will appear in *JAOAC* (1977) March issue.

This report of the Associate Referee, J. O. Watts, was presented at the 90th Annual Meeting of the AOAC, Oct. 18–21, 1976, at Washington, DC.

Received April 21, 1976.

Table 1. Methyl mercury in fish and shellfish with recovery data for added methyl mercury chloride (MMC) and ethyl mercury chloride (EMC)

Sample	Methyl mercury found (as ppm Hg)	Recovery, %			
		0.50 ppm added		0.10 ppm added	
		MMC	EMC	MMC	EMC
Skipjack tuna (canned)	0.09	98	99	108	100
	0.09	98	98	105	100
	0.09			102	100
	0.10			102	97
Mean±SD[a]	0.093±0.005	98.0	98.5	104.3±2.9	99.3±1.5
Albacore tuna (canned)	0.48	100	98	ND[b]	ND
	0.47				
	0.46				
	0.45				
	0.45				
	0.45				
Mean±SD	0.460±0.013	100	98	ND	ND
Bluefin tuna (canned)	0.87	99	94		
	0.84	98	94		
Mean	0.855	98.5	94.0	ND	ND
Cod (fresh)	0.15	97	101	100	100
	0.16	100	105	95	100
Mean	0.155	98.5	103.0	97.5	100.0
Mackerel (fresh)	0.07	98	100	102	102
	0.07	98	99	100	100
Mean	0.07	98.0	99.5	101.0	101.0
Shrimp (fresh frozen)	0.04	97	97	97	96
	0.04	98	98	94	94
	0.03			96	96
	0.03			97	96
Mean±SD	0.035±0.006	97.5	97.5	96.0±1.4	95.5±1.0
Lobster tail (frozen)	0.03	97	106	99	97
	0.03	100	108	108	95
	0.02			104	96
	0.02				
Mean±SD	0.025±0.006	98.5	107.0	103.7±4.5	96.0±1.0
Lobster claw (cooked)	0.27	105	100		
	0.27	103	100		
Mean	0.27	104.0	100.0	ND	ND
Lobster tail (cooked)	0.76	101	99		
	0.77				
	0.75				
	0.76				
Mean±SD	0.76±0.008	101	99	ND	ND
Oyster	0.01	97	97	98	IP[c]
	<0.01			98	
	0.01			97	
	<0.01				
	0.01				
	<0.01				
	<0.01				
Mean±SD	<0.01	97	97	97.7±0.6	ND

[a] SD = standard deviation.
[b] ND = not determined.
[c] IP = not determined due to interfering peak adjacent to EMC.

peaks from the added mercury compounds superimposed on the background.

Because fish and shellfish samples do not usually contain ethyl mercury, spiking the sample with ethyl mercury chloride provides a good internal standard if a replicate analysis of the unspiked sample shows that no interfering peaks are present. Poor recovery of this compound

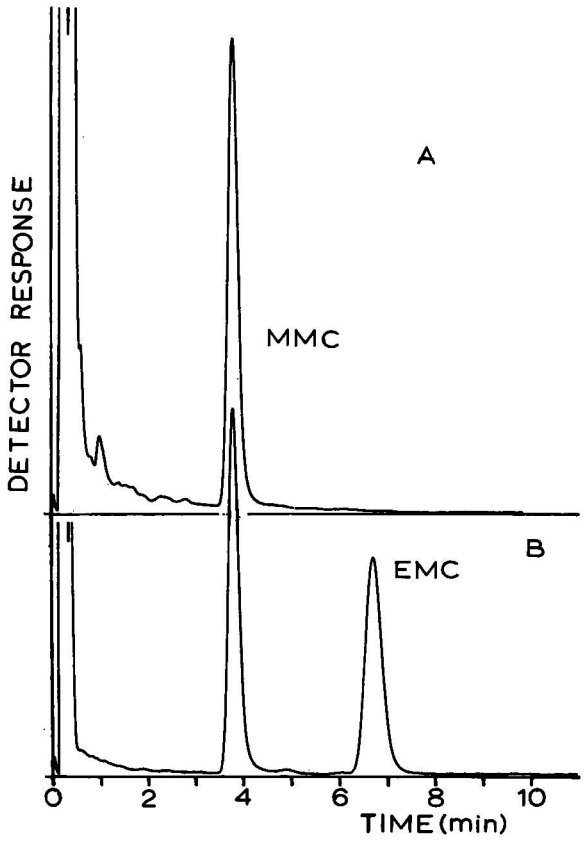


FIG. 1—Typical gas chromatograms with clean background (recorder scale expansion 16×): A, unspiked tuna sample with 0.65 ppm methyl mercury chloride (MMC) (0.65 ng injected); B, standard solution containing 0.5 ppm each of MMC and ethyl mercury chloride (EMC) (0.50 ng of each injected).

could indicate analyst error or some unsuspected instrumental problem.

The 4′ glass GLC column packed with 15% DEGS on Gas-Chrom P provided excellent separation of methyl and ethyl mercury chloride at a column temperature of 160°C. Earlier work with a 6′ column at temperatures between 175 and 190°C yielded GLC peaks with good separation and shorter retention times. Unfortunately, column bleed was significant at the higher temperatures, causing a decrease in detector sensitivity. In addition there was a gradual decrease in retention times with eventual column failure. Lowering the column temperature to 160°C resulted in a marked increase in detector sensitivity. Although retention times are slightly increased, column life is greatly extended. With column temperatures and carrier gas flow rate held constant, the retention time decrease due to substrate bleed-off was only 2% during a 6-month period. A commercial "pretested" DEGS column packing was used in the present work, while a "self-prepared" batch was used for earlier work. Both were satisfactory.

The gas chromatograph used was equipped with a constant current electron capture detector. Peak height response was checked for 5 μl volumes containing between 0.02 and 2.0 ppm (based on 5 g sample extract in 25 ml benzene) of methyl and ethyl mercury chloride prepared by appropriate dilution of the standard solutions. The response was found to be linear with zero

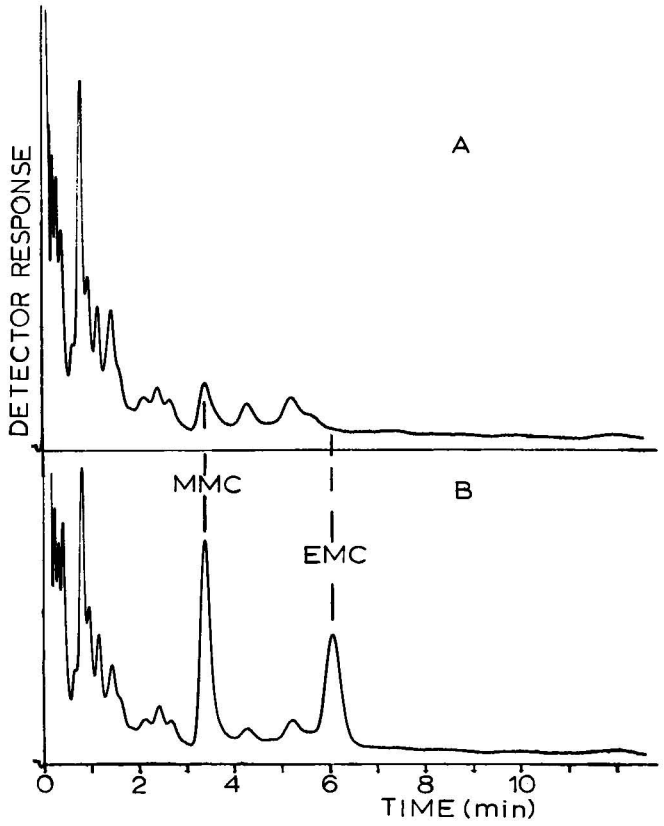


FIG. 2—Typical gas chromatograms with limiting background (recorder scale expansion 8×): A, unspiked lobster sample; B, lobster sample A spiked with 0.1 ppm each of methyl mercury chloride (MMC) and ethyl mercury chloride (EMC).

intercept; thus calculations were based on direct comparison of peak heights obtained for standard and sample solutions. Continuous use of the ^{63}Ni electron capture detector at 350°C resulted in no detrimental effects and eliminated the need for periodic cleaning.

In general the sample cleanup provided by this method is adequate for the quantitative GLC determination of methyl and ethyl mercury chloride in fish and shellfish at levels above 0.10 ppm. When a higher detector sensitivity is used for levels below 0.10 ppm, extraneous background peaks begin to dominate the chromatograms, so that interpretation of small peaks as methyl or ethyl mercury chloride becomes questionable. With an exceptionally clean background the detection limit for both compounds, based on a 5 g sample extract in 25 ml benzene, is about 0.01 ppm for a 5 μl injection. If additional concentration of the sample solution is necessary to increase sensitivity, an additional cleanup step will be needed.

One problem encountered was that a gel can be formed in the benzene layer in the centrifuge tubes. During the analysis of tuna fish samples, gel emulsion formation containing entrapped benzene occurred occasionally in the benzene phase adjacent to the aqueous phase after centrifugation to separate the acidified aqueous and organic benzene phases. In these instances it was necessary to gently shake the solution and recentrifuge. For lobster, shrimp, and oyster sam-

ples, severe gel formation prevented removal of benzene even after repeated shaking and centrifugation. Adding 5 ml isopropanol to the acidified aqueous layer prior to benzene extraction eliminated gel formation for all sample types except oysters. In the case of oysters gel formation also occurred, but only during the final 2 benzene extractions. By adding an additional 1 ml isopropanol prior to each of these last 2 benzene extractions, the gel formation problem was also eliminated for oysters.

Concentration of the combined benzene extraction solutions in the Kuderna-Danish apparatus over a steam bath was difficult when the conventional, 3-bubble chamber Snyder column was used. Most of the benzene vapor condensed before it could exit from the column. After the top bubble chamber was cut off, the benzene vapor was easily driven out of the apparatus under steam bath conditions. An 85 ml benzene volume could be concentrated to 8 ml in about 15 min.

Unlike the Westöö methods (6, 7), the method described results in essentially total extraction of methyl mercury and ethyl mercury. Therefore, no recovery corrections are required. At methyl and ethyl mercury chloride levels of 0.1 ppm the method also has the advantage over the Westöö methods of not requiring the additional cysteine cleanup step. The acetone and benzene sample prewash removes most interfering organics with no loss of methyl mercury or ethyl mercury. Complete bonding of methyl mercury and ethyl mercury to the protein of tuna fish was confirmed from recovery experiments in which standard solutions were filtered through the sample. GLC analyses of the filtrate showed a large solvent front followed by many extraneous peaks, but none with retention times corresponding to methyl or ethyl mercury chloride. Apparently all the methyl and ethyl mercury rapidly bonds to the protein matrix. Subsequent treatment with dilute hydrochloric acid quantitatively hydrolyzes the organomercury-protein complex, releasing the added methyl and ethyl mercury chloride.

The method described for quantitative determination of methyl and ethyl mercury chloride at 0.1 ppm in fish and shellfish provides a more rapid procedure with fewer sample manipulations than other methods currently being used. Because of the general absence of ethyl mercury in fish and shellfish in which methyl mercury is to be determined, ethyl mercury chloride can serve as an internal standard to detect unsuspected error and changing instrumental conditions, unless analysis of unspiked samples shows the presence of peaks which interfere with those for ethyl mercury chloride.

Acknowledgment

The authors wish to thank John Paullin, National Canners Association, Washington, DC, who conducted a method trial of the experimental procedure.

REFERENCES

(1) Jensen, S., & Jernelov, A. (1969) *Nature London* **223**, 753–754
(2) Wood, J. M., Kennedy, F. S., & Rosen, C. G. (1968) *Nature London* **220**, 173–174
(3) "Hazards of Mercury," Special Report to the Secretary's Pesticide Advisory Committee, Department of Health, Education, and Welfare (1971) *Environ. Res.* **4**, 1–69
(4) Yamanaka, S., & Ueda, K. (1975) *Bull. Environ. Contam. Toxicol.* **14**, 409–414
(5) Schafer, M. L., Rhea, U., Peeler, J. T., Hamilton, C. H., & Campbell, J. E. (1975) *J. Agric. Food Chem.* **23**, 1079–1083
(6) Westöö, G. (1966) *Acta Chem. Scand.* **20**, 2131–2137
(7) Westöö, G. (1967) *Acta Chem. Scand.* **21**, 1790–1800
(8) Uthe, J. F., Solomon, J., & Grift, B. (1972) *JAOAC* **55**, 583–589
(9) Magos, L. (1971) *Analyst London* **96**, 847–853
(10) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, secs. **25.103–25.105**
(11) *Fed Regist.* (Dec. 6, 1974) **39**(236), 43738–43740
(12) Munns, R. K., & Holland, D. C. (1971) *JAOAC* **54**, 202–205

Cathodic Stripping Voltammetry of Nanogram Amounts of Selenium in Biological Material

MICHAEL W. BLADES, JOHN A. DALZIEL, and CLIVE M. ELSON
Department of Chemistry, Saint Mary's University, Halifax, Nova Scotia, Canada B3H 3C3

A method is described to determine selenium in biological material, based on cathodic stripping voltammetry. Following wet ashing, the selenium was extracted into benzene as the 3',4'-diaminophenylpiazselenol. The selenium was subsequently back-extracted into dilute acid for analysis. Analyses of NBS Bovine Liver demonstrated that the method was capable of recovering 96±9% of the selenium present. The detection limit and working range were 3 ng/g and 3–10,000 ng/g, respectively. The method was also applied to the determination of selenium in rapeseed oils and seed.

It is well known that selenium in certain forms is toxic to man and animals. However, it has been established that selenium is also an essential micronutrient for plants and animals (1, 2). This dichotomy of behavior depends on both the level of selenium intake and the form of the selenium. Since certain plants are known to accumulate selenium and since selenium and sulfur cannot always be distinguished by enzyme systems (1), it is essential that the concentration of selenium in foodstuffs be established.

The method recommended for the determination of selenium in plants and foods (3) involves reacting Se(IV) with an aromatic *ortho*-diamine (e.g., 2,3-diaminonaphthalene), extracting the resultant piazselenol into an organic solvent, and measuring the fluorescence. This method suffers from interferences by copper (4), iron (5), and strong oxidizing and reducing agents; high reagent blanks (6); and the instability of the piazselenol which requires that the fluorescence measurement be made immediately (within 5 min) after extraction (6). A collaborative study employing a modified fluorometric method has recently been reported by Ihnat (7).

Recently, selenium has been studied by both anodic (8) and cathodic (9) stripping voltammetry. In the latter case, Se(IV) was reported to interact with a mercury electrode in the presence of chloride ion to form a complex which was stripped from the surface of the electrode at cathodic potentials. This report describes a method for the determination of selenium over the range 3–10,000 ng, using cathodic stripping voltammetry, and its application to the analysis of proteinaceous materials and oils.

METHOD

Apparatus

(a) *Digestion apparatus.*—50 ml micro-Kjeldahl digestor equipped with glass manifold (Electrothermal Engineering Ltd., London, England) and 30 ml long-neck Pyrex flasks.

(b) *Wrist-action shaker.*—Burrell Corp.

(c) *Stripping analyzer.*—Polarographic analyzer (Model 174, Princeton Applied Research Corp., Princeton, NJ) coupled to x-y recorder (Omnigraphic 2000, Houston Instruments Ltd.). The reference electrode was a low leakage, saturated NaCl calomel electrode; the counter electrode was a piece of platinum wire; and the working electrode was a hanging mercury drop micrometer electrode. The polarographic cell was made of borosilicate glass (supplied by Princeton Applied Research Corp., accessory 9301) and had a working volume of 5–50 ml. A thermostated cell is preferable.

Reagents

All solutions were prepared in glass-distilled water. Glassware was cleaned with $KMnO_4/H_2SO_4$ mixture. All acids and bases were ultra high purity grade (Ultrex grade, J. T. Baker Chemical Co., Phillipsburg, NJ) unless otherwise specified.

(a) *Ethylenediaminetetracetic acid (EDTA) solution.*—0.1*M*. Dissolve 3.72 g disodium salt (Fisher reagent grade) in water and dilute to 100 ml.

(b) *3,3'-Diaminobenzidine hydrochloride (DAB) solution.*—1.2% w/v. Dissolve 0.12 g DAB (99.0% minimum, J. T. Baker Chemical Co.) in 10 ml 1*N* $HClO_4$. Extract 15 min with 10 ml cyclohexane with shaking and discard cyclohexane. Prepare solution daily and store in dark. DAB which is 6 months old is not recommended for use, since it gives high background signals.

(c) *Back-extraction mixture.*—6 ml water plus 0.4 ml $HClO_4$ in 50 ml Erlenmeyer flask.

(d) *Selenium stock solution.*—1000 ppm. Dissolve 1.6497 g H_2SeO_3 (99%, Alfa Products, Beverly, MA) in 1 L 0.1*N* HCl. Serially dilute to

prepare 1 and 10 ppm solutions in 0.1*N* HCl. The selenous acid was supplied in crystalline form in a sealed vessel containing argon and was stored in a desiccator. The acid was used directly but other standards such as selenium metal (3) may also be used.

(e) *Benzene.*—Fisher Certified ACS (thiophene-free).

(f) *Nitrogen.* — High purity nitrogen was scrubbed by using reduced BASF catalyst (R311, BASF Corp., Parsippany, NJ) at 100°C and then saturated with the electrolyte.

Preparation of Samples

The bovine liver samples (NBS Standard Reference Material 1577, selenium content 1.1±0.1 ppm) were freeze-dried 10 hr at 0.01 mm Hg and −60°C. The oils (rapeseed oils from Rapeseed Association of Canada; soybean oil from Canada Packers Ltd.) were stored in a refrigerator under nitrogen and were analyzed directly. Samples of rapeseed were crushed lightly in a glass mortar and pestle but were not dried.

Calibration Curves

The curves were prepared by determining the stripping peak current (*see* below) at various concentrations of selenium. The stock selenium solutions were diluted using either 0.2*N* HCl plus 0.3*N* $HClO_4$ for concentrations of 0–8 ppb or 0.2*N* HCl for the range 20–500 ppb selenium and analyzed directly without further treatment.

Determination

Accurately weigh ca 0.3 g bovine liver, 1 g oil, or 0.8 g seed directly into Kjeldahl flask and add 6 ml HNO_3 plus glass anti-bumping tube. (Reagent grade acids were also suitable for the digestion step, since the selenium was subsequently isolated.) Heat mixture on digestion unit at setting that causes acid to reflux in neck of flask. (*Note:* Samples froth vigorously during initial stages of heating.) Continue digestion until NO_2 fumes cease to appear (for liver samples, this requires ca 5–6 hr, but oils may take 24–48 hr). Add 3 ml $HClO_4$ and increase temperature to evaporate water and HNO_3. If charring occurs, immediately add 2 ml HNO_3 and reheat 2–3 hr before evaporating HNO_3 again. After fumes of $HClO_4$ appear, continue heating 20–30 min. Cool, and transfer digest to beaker with the aid of water and disposable pipets.

Add 0.5 ml HCl and bubble oxygen through the solution 5–10 min. (Oxygen is used to destroy reducing agents but gentle boiling should accomplish same result.) Adjust pH to 2–3 with NH_4OH and monitor with pH meter. Add 1 ml EDTA solution. (If pH increases much above 3 before EDTA is added, metal hydroxides may precipitate and remove selenium from solution.) Stir solution 5 min. Add 0.2 ml freshly prepared DAB solution and stir 1 hr. A shorter period might be used if solution is warmed to assist complex formation (6). Adjust pH to 7 and transfer solution to 100 ml separatory funnel equipped with Teflon stopcock. Sample volume is ca 25 ml at this stage, including washings.

Extract sample with two 10 ml portions of benzene for 15 min each, using wrist-action shaker. (The efficiency of the first extraction or the optimum volume of benzene was not determined.) After first extraction, wait 10 min and then transfer most of the benzene layer to Erlenmeyer flask containing back-extraction mixture. Stir benzene plus back-extraction mixture as second extraction proceeds. Discard aqueous layer and transfer second portion of benzene to the Erlenmeyer flask. Stir mixture for additional 10 min and then place flask in water bath and slowly bring water to boil. After benzene has been driven off, continue heating additional 5 min. Cool solution and transfer to volumetric flask (for samples containing <80 ng selenium, use 10 ml flask, and for liver samples, use 50 or 100 ml flask). Adjust HCl and $HClO_4$ concentrations to match those of solutions used in preparing calibration curve.

Introduce sample solution into polarographic cell and insert electrodes. De-aerate for 15 min, using nitrogen, and then direct gas stream over solution. Ensure that all gas bubbles are removed from tip of capillary of mercury electrode. Extrude mercury drop of ca 2.8 mg (3 divisions on barrel of micrometer electrode). Stir solution (Magnestir, Canlab, with separate on-off switch to reproduce stirring rate), using 14 × 4 mm magnetic bar at rate such that no turbulence is seen in solution (ca 250 rpm). Synchronous motor stirrer is preferable. Deposit selenium at +0.05 v for 2 min if sample solution concentration is 0–8 ppb and for 1 min if concentration is >20 ppb. After initial deposition period, turn stirrer off and wait 30 sec before commencing potential scan. For dilute solutions, use differential pulse mode to measure reduction currents. Typical instrument settings were: cathodic scan 5 mv/sec, pulse modulation amplitude 50 mv, droptime 1 sec, and current sensitivity 0.5 μa full scale. For samples in the range 20–500 ppb, direct current cathodic stripping voltammetry was employed using scan rate of 20 mv/sec, and current sensitivities of 0.2–5.0 μa full scale. Three voltammograms were recorded for each sample and an average current reading was taken. *Range* of 3 results should not exceed 0.03 μa for settings used in pulse mode. Variations larger than

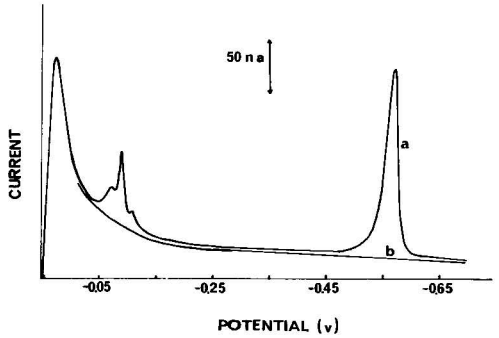


FIG. 1—Direct cathodic stripping voltammograms of a, 25 ml sample of 56 ppb Se(IV) in 0.2*N* HCl; and b, 25 ml sample of 0.2*N* HCl. Peak current measured at −0.56 v.

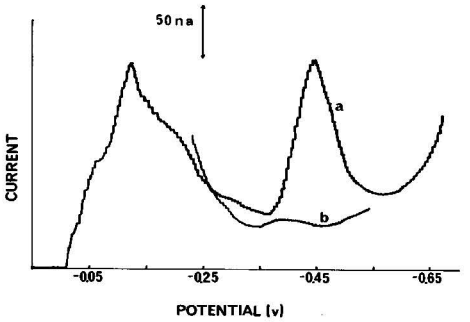


FIG. 2—Differential pulse cathodic stripping voltammograms of a, sample solution containing 3.9 ng Se/ml in 0.2*N* HCl/0.3*N* $HClO_4$ derived from bovine liver standard; and b, reagent blank in 0.2*N* HCl/0.3*N* $HClO_4$. Peak current measured at −0.44 v.

this usually result from malfunction of mercury electrode.

Correct stripping currents for reagent blanks by placing same amount of HNO_3 and $HClO_4$ required to digest sample in Kjeldahl flasks and repeat above procedure.

Results and Discussion

The electroactive form of selenium for the purposes of this study was Se(IV); hence HCl was added after the sample digestion and again after the back-extraction step to reduce Se(VI) to Se(IV). The direct cathodic stripping voltammograms of solutions containing 20–500 ppb exhibited 2 reduction peaks at −0.08 and −0.56 v (Fig. 1). The first reduction step involves the conversion of an adsorbed chloro selenium complex to mercuric selenide. The mercuric selenide is then further reduced at more cathodic potentials to mercury and hydrogen selenide (7). The wave at −0.08 v had a complex structure which was dependent on the concentration of selenium and interfered with the measurement of peak current. Nevertheless, the stripping currents were approximately proportional to concentration. The wave at −0.56 v was well defined and a plot of peak current vs. concentration was linear over the range studied (20–500 ng/ml, corresponding to 0.5–12.5 μg selenium in 25 ml sample) which did not represent the limits of applicability of the technique. The equation of the least squares line was $i = 3.03 \pm 0.03 \times 10^{-3}C - 1.56 \pm 1.10 \times 10^{-2}$ (μa), where C is in units of ng selenium/ml and the correlation coefficient of the fit was 1.000. Olson (6) and Ihnat (7) reported upper limits of the fluorometric technique as 0.4 and 0.8 μg selenium, respectively.

For solutions containing less than 10 ng selenium/ml, the technique of differential pulse stripping voltammetry was employed. A typical voltammogram is shown in Fig. 2. The region between +0.05 and −0.05 v displayed a large cathodic current due to the reduction of mercurous chloride which partially obscured the first selenium stripping wave; hence, this region of the voltammogram was scanned at a reduced sensitivity. The major reduction peak appeared at −0.44 v and the peak height was directly proportional to concentration over the range 0–8 ppb (Fig. 3). The electrolyte was a mixture of $HClO_4$ and HCl, because perchloric acid enhances the height of the wave and improves its definition.

The instrumental settings for the direct and differential pulse cathodic stripping techniques were chosen to optimize both the size of the peak current and the reproducibility. General considerations relating to these techniques have been reported by Flato (10). The mercury micrometer electrode gives more reproducible results for drops formed by using 3 divisions on the barrel rather than 6. The number of divisions recommended by the supplier is generally in the range 3–6 divisions. The initial potential was dictated by the formation of the mercury-selenium-chloride complex and mercurous chloride: The selenium complex will not form at cathodic potentials and very anodic potentials

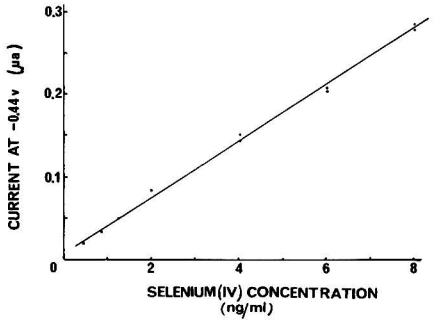


FIG. 3—Calibration plot of peak current vs. concentration of Se(IV) for the range 0–8 ng/ml.

will generate sufficient mercurous chloride to mask the stripping peaks. The deposition time was long enough to form a detectable amount of selenium complex but sufficiently short to prevent "instrumental instabilities" (10) from limiting the reproducibility. A scan rate of 5 mv/sec provided a reasonable analysis time and allowed the system to remain in equilibrium with the electrode, thus maximizing the reproducibility. The pulse modulation amplitude was determined by preparing calibration curves over the range 0–10 ppb selenium. The curve prepared using a 50 mV pulse provided the best compromise between large currents and closest approximation to a straight line.

The plot of Fig. 3 was the working curve for all the analyses and combines the results of 2 separate calibrations performed on consecutive days. A detection limit of 0.31 ppb at 95% confidence limit was determined by the method of Skogerboe and Grant (11), using the pooled standard deviation of the current residuals from the least squares line. The detection limit corresponded to 3 ng selenium in a 10 ml sample. However, selenium levels of 0.1 and 0.2 ng/ml did exhibit signals which were barely discernible. The calibration curve was duplicated on separate occasions and, hence, the above curve was used over a period of several months because the reagents and apparatus were not changed during this time. Different laboratory workers reproduced the results to within the uncertainties quoted below over 6 months.

The above procedure was introduced after attempts to analyze the digested samples directly were unsuccessful. The selenium peaks were lost in the high background signals due to the reduction of mild oxidizing agents such as ferric and cupric ions. The procedure and working curves were tested by analyzing 5 samples of NBS Bovine Liver; the results appear in Table 1. The peak currents have been corrected for reagents blanks which displayed a reduction peak at −0.44 v, corresponding to 0.2 ppb selenium in a final volume of 10 ml. The average per cent recovery was 96±9% with all experimental values falling within ±10% of the theoretical values. The same per cent recovery was obtained when sample solutions of the liver standard were analyzed at the 40 ppb level.

The high percentage recovery confirmed that the extractions were very efficient and that the warm perchloric acid solution used in the back-extraction decomposed the piazselenol and released all the selenium. To ensure that the back-extraction was successful, benzene was used in place of the usual solvents, decalin and cyclohexane (4, 6). Simple back-extractions into 2*N* $HClO_4$ without the evaporation of the organic solvent gave erratic results with losses as high as 30%. In an attempt to simplify the method, selenium was extracted with 2,3-diaminonaphthalene (DAN) and cyclohexane from aqueous solutions of pH 2. However, the voltammogram had higher background signals, presumably due to the greater stability of DAN.

Table 1. Analysis of standard bovine liver

Sample wt, g	Calcd Se concn, ppb	Corrected peak current, μA	Se concn found, ppb	Rec., %
0.2710	5.96 (50 ml)[a]	0.23	6.3	106
0.4048	4.45 (100 ml)	0.15	3.9	87
0.2859	6.29 (50 ml)	0.23	6.5	104
0.2925	6.44 (50 ml)	0.22	6.2	97
0.2580	5.68 (50 ml)	0.18	4.9	87
			Av.	96±9

[a] Final volume.

Table 2. Analysis of oils and seed

Sample	Se content, ng/g
Crude regular rapeseed oil	8.9±2.5
Fully refined regular rapeseed oil	2.8±1.8
Crude Tower variety rapeseed oil	7.9±3.8
Fully refined Tower variety rapeseed oil	<1
Desolvenated Target variety rapeseed oil	3.8±1.3
Crude soybean oil	<1
Tower variety seed	70±12

The method appeared to be free from interferences since the liver standard contained many elements, some of which were at levels 200–300 times that of selenium (e.g., iron and copper). The method of selenium analysis which Shendrikar (4) reported that the U.S. Environmental Protection Agency recommends involves an initial extraction of selenium from aqueous phase prior to digestion and fluorometric analysis. Hence the method reported here offers several advantages.

The results of the analysis of the oils and seed appear in Table 2. All samples were analyzed in triplicate and the uncertainties are expressed at the 95% confidence level. The range of the results was always less than 3 ng selenium/g and the uncertainties reflect the limited number of measurements made. The 2 crude oils had similar selenium contents despite the fact that Tower oil was derived from a recently developed variety of *Brassica napus* which yields oil with a low erucic acid content and meal with a low glucosinolate content, whereas the other crude oil came from the older "regular" type of rapeseed. However, upon refining (12), which includes degumming with dilute phosphoric acid, alkali washing, bleaching with activated earths, and deodorizing by steam distillation, the Tower variety oil released more selenium than did the regular oil. Refining has also been reported to reduce the sulfur content of rapeseed oils from approximately 25 ppm to less than 1 ppm (13, 14). The sample of Target oil was derived from a variety of *B. napus* which contained the normal level of erucic acid. The oil was extracted and refined by J. D. Jones of Food Research Institute, Agriculture Canada, Ottawa. The oil was also analyzed by the Analytical Chemistry Services of the Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, by using the fluorometric method (3) and contained 12 ng selenium/g. By comparison with the rapeseed oils, the selenium content of the crude soybean oil was at least one order of magnitude smaller.

Samples of the Tower variety seeds contained 70 ng selenium/g. Since Tower variety rapeseeds are approximately 42% oil and 45% protein (15) and since the crude oil contained only 7.9 ng selenium/g, the selenium content of meals derived from the protein portion of the seeds must be 0.15 μg selenium/g. This value is considerably smaller than that reported for meals derived from rapeseeds of the *B. campestris* species grown in Canada and is comparable to the selenium content of meals derived from *B. napus* types grown in Europe (16).

The results of the present work together with those of Hougen and Daun (13, 14) suggest that the sulfur content of oils can be estimated from the level of selenium by using a ratio of $1–4 \times 10^3$. The ratio of sulfur to selenium in geological samples was reported to be $5–10 \times 10^3$ (17).

Conclusion

The method described with minor modifications offers several advantages over other methods for the determination of selenium: wider working range, low reagent blanks allow a few nanograms to be detected, no known interferences, and the instability of a piazselenol is used to advantage during a back-extraction step. Furthermore, because selenium is separated from the digestion matrix prior to analysis, it is assumed that the method will apply equally well to other types of samples (e.g., geological samples, metals, and alloys).

Acknowledgments

The authors wish to thank the Rapeseed Association of Canada, Winnipeg, Manitoba, for financial support and H. Siegerman, Princeton Applied Research Corp., Princeton, NJ, and R. G. Ackman, Fisheries and Marine Services, Halifax Laboratory, Nova Scotia, for their helpful suggestions and advice. The technical assistance of B. Wells is also gratefully acknowledged.

References

(1) Stadtman, T. C. (1974) *Science* **183**, 915–922
(2) Frost, D. V. (Jan. 24, 1972) *Feedstuffs* 8

Received March 31, 1976.

(3) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, secs. **3.074–3.078, 25.117–25.120**
(4) Shendrikar, A. D. (1974) *Sci. Total Environ.* **3**, 155–168
(5) Clarke, W. E. (1970) *Analyst* **95**, 65–69
(6) Olson, O. E. (1969) *JAOAC* **52**, 627–634
(7) Ihnat, M. (1974) *JAOAC* **57**, 373–378
(8) Andrews, R. W., & Johnson, D. C. (1975) *Anal. Chem.* **47**, 294–299
(9) Vajda, F. (1970) *Acta Chim. Acad. Sci. Hung.* **63**, 257–265; thru *Chem. Abstr.* (1970) **72**, 106806*u*
(10) Flato, J. B. (1972) *Anal. Chem.* **44**, 75A–87A
(11) Skogerboe, R. K., & Grant, C. L. (1970) *Spectrosc. Lett.* **3**, 215–217
(12) Appleguist, L. A., & Ohlson, R. (Eds.) (1972) *Rapeseed,* Elsevier Publishing Co., Amsterdam, pp. 218–248
(13) Hougen, F. W., & Daun, J. K. (June 1975) *Progress Report to Rapeseed Association of Canada,* Manitoba
(14) Daun, J. K. (1973) M.S. Thesis, University of Manitoba, Manitoba
(15) Downey, R. K., Klassen, A. J., & McAnsh, J. (1974) *Rapeseed,* Rapeseed Association of Canada, Manitoba, No. 33
(16) Blair, R., & Scougall, R. K. (Feb. 10, 1975) *Feedstuffs* 26–27
(17) Lavrakas, V., Golembeski, T. J., Pappos, G., & Gregory, J. E. (1974) *Anal. Chem.* **46**, 952–954

MEAT AND MEAT PRODUCTS

Determination of Added Blood in Ground Beef

ALBERT B. KARASZ[1], ROSS ANDERSEN, and ROGER POLLMAN
New York State Food Laboratory, Department of Agriculture and Markets, Albany, NY 12235

Blood added to ground beef is estimated from hemoglobin extracted with water. Since myoglobin is co-extracted with the hemoglobin, the 2 heme pigments are separated in one portion of the extract by precipitating the hemoglobin in an 85% $(NH_4)_2SO_4$ solution. The myoglobin remaining in solution is used as the reference solution for determining the hemoglobin in another portion of the extract containing both pigments. The hemoglobin is converted to cyanomethemoglobin and quantitated by its absorbance at 422 nm.

Blood is added to ground beef to conceal the presence of excess fat, to enhance the color, or simply as an extender. One method for the determination of added blood has been described by Hankin (1) and is based on the amount of iron extracted from the sample by water. However, the method is time consuming, employs a noxious reagent, mercaptoacetic acid, and uses in the computation a blank value which is high because it represents the iron from both the hemoglobin and the myoglobin released from the tissue during grinding.

An improved method, based on the most widely used clinical procedure for measuring hemoglobin in blood (2), estimates added blood from the hemoglobin extracted from ground beef. In this procedure, hemoglobin is oxidized by ferricyanide to methemoglobin and is complexed with cyanide to form cyanomethemoglobin which is measured colorimetrically. A standard reagent and a certified standard, both available commercially, are used to quantitate the hemoglobin.

Modifications are necessary for applying this procedure to ground beef because hemoglobin is diluted when extracted from the sample with water, and the wavelength used in the clinical procedure, 540 nm, is not sensitive enough. Cyanomethemoglobin can be measured with much greater sensitivity at 422 nm, as shown in Fig. 1.

[1] Retired.

However, ferricyanide, which is used in the clinical method, absorbs at 422 nm and is subject to interference from reducing agents such as ascorbic acid. Therefore, a modified reagent, substituting sodium nitrite as the oxidizing agent, is used. This does not alter the validity of the standard curve which is prepared from the certified standard and the clinical reagent.

Both the commercial and the modified reagents react with myoglobin to form cyanometmyoglobin which has the same absorption properties as cyanomethemoglobin (Fig. 1). This interference is eliminated by separating the 2 heme pigments by their differential solubilities in $(NH_4)_2SO_4$ solution (2). In an 85% saturated $(NH_4)_2SO_4$ solution, myoglobin is soluble but hemoglobin is precipitated. Two different assay solutions can be prepared from the meat extract: one containing myoglobin only, the other containing both myoglobin and hemoglobin. The difference in absorption between the 2 solutions is a measure of the hemoglobin content.

The hemoglobin found is a measure of both

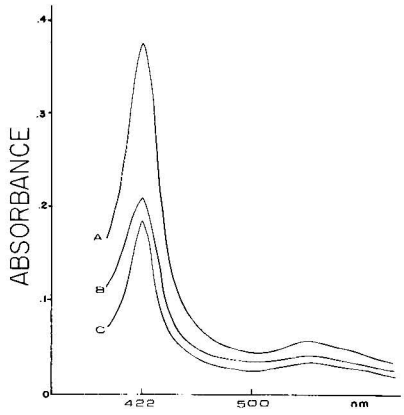


FIG. 1—Absorption spectra of cyanomethemoglobin and cyanometmyoglobin extracted from ground beef: A, cyanomethemoglobin and cyanometmyoglobin in water; B, cyanometmyoglobin in $(NH_4)_2SO_4$ solution; C, cyanomethemoglobin, obtained by scanning A against B.

the added and the normal residual blood in the ground beef. A reasonable approximation of normal residual hemoglobin in ground beef was established so that this value could be deducted from the total hemoglobin found in the sample. The difference is a measure of the blood added to ground beef.

METHOD

Reagents and Apparatus

(a) *Cyanomethemoglobin reagent.* — Hycel Corp., 7920 Westpark Dr, Houston, TX 77036; prepare as directed by manufacturer.

(b) *Cyanomethemoglobin standard.*—Certified 0.80 mg hemoglobin/ml (Hycel Corp).

(c) *Ammonium sulfate solution.*—Prepare 1.5 L saturated $(NH_4)_2SO_4$ solution. Filter through glass wool.

(d) *Cyanide reagents.*—Caution: *See* sec. 51.050 (3). *Cyanide reagent A.*—Dissolve 3.403 g KH_2PO_4, 50 mg KCN, and 350 mg $NaNO_2$ in water and dilute to 1 L with water. *Cyanide reagent B.*—Dissolve 3.403 g KH_2PO_4, 4.355 g K_2HPO_4, 50 mg KCN, and 350 mg $NaNO_2$ in 40 ml water in 1 L volumetric flask. Add 944.5 ml saturated $(NH_4)_2SO_4$ solution (c) and adjust to volume with water. This solution, as used in the method, will provide a solution 85% saturated with respect to $(NH_4)_2SO_4$.

(e) *Filter paper.*—Glass fiber, 7 cm diameter (Reeve Angel, Grade 934 AH, or equivalent).

(f) *Spectrophotometer.*—Beckman DB, or equivalent.

Preparation of Standard Curve

Using cyanomethemoglobin standard and cyanomethemoglobin reagent make dilutions equivalent to 0.00, 0.004, 0.08, 0.12, and 0.16 mg hemoglobin/ml. These concentrations are equivalent to 0, 200, 400, 600, and 800 mg hemoglobin/100 g sample of ground beef, respectively. Using 0.00 dilution as blank, determine absorbances of other solutions at 422 and 500 nm. Plot net absorbance $A = A_{422} - A_{500}$ against mg hemoglobin/100 g sample.

Determination

Do *not* regrind ground beef. Place entire sample, including any liquid present, in plastic bag and mix thoroughly by kneading. Weigh 10.00 g sample into 100 ml beaker. Add 10 ml water; break up and slurry sample with glass rod. Add 40 ml water and let stand 15 min, stirring occasionally. Filter through loose wad of glass wool. Pipet 1 ml aliquot into 10 ml volumetric flask. Dilute to volume with cyanide reagent A (solution A). Pipet 1 ml aliquot into second 10 ml volumetric flask. Dilute to volume with cyanide reagent B (solution B). Filter both solutions through glass fiber paper.

Zero spectrophotometer against air and read solution A, using solution B as the reference, at 422 and 500 nm. From net absorbance and standard curve, determine the concentration of hemoglobin per 100 g sample. Calculate added blood, using the following equation:

$$\text{Added blood, \%} = (\text{mg hemoglobin found/100 g sample} - 171 \text{ mg hemoglobin/100 g meat})/107 \text{ mg hemoglobin/g blood}$$

where 171 mg hemoglobin/100 g is the average residual hemoglobin content of unadulterated ground beef and 107 mg hemoglobin/g blood is the average amount of hemoglobin recovered from 1 g added blood.

Results and Discussion

Ground beef naturally contains some residual hemoglobin. To determine this value, 25 samples of unground stewing beef were collected from as many sources. The samples were ground and hemoglobin was determined by the proposed method. The myoglobin extracted was also determined from the absorbance of the ammonium sulfate solution referenced against a reagent blank. Fat was estimated by using the Paley bottle method of Salwin *et al.* (4) to determine changes in the hemoglobin and myoglobin concentrations with fat content. The results indicated that hemoglobin concentration was fairly constant, averaging 176 mg/100 g meat, while myoglobin increased as fat decreased.

An additional 78 surveillance samples of ground beef were examined for hemoglobin by the proposed method. Fat content was estimated in all samples and myoglobin was determined on 60 of the samples. The combined data from the 2 series of tests were broken into subsets based on fat content and are shown in Table 1.

Hemoglobin is fairly constant over the range of fat normally encountered in ground beef samples while myoglobin increases as fat decreases. The average hemoglobin content or meat blank for ground beef was calculated as 171 mg hemoglobin/100 g meat.

Recoveries of hemoglobin were measured by using ground beef samples of different fat contents which had been enriched with bovine blood at levels of 0–4%. The blood was stabilized with EDTA and the hemoglobin was determined along with the enriched samples. Recoveries of added hemoglobin averaged 88.7% and

Table 1. Hemoglobin and myoglobin (mg/100 g meat) extracted from ground beef

	Hemoglobin				Myoglobin			
% Fat	No. of samples	Range	Av.	Std dev.	No. of samples	Range	Av.	Std dev.
0–10	9	141–215	169.8	24.05	8	178–260	219.0	33.77
10–15	15	141–205	176.5	22.40	15	109–245	179.1	41.87
15–20	18	146–210	181.2	20.39	18	82–263	154.8	46.87
20–25	21	128–226	170.4	24.91	16	96–191	138.8	31.24
25–30	26	119–191	164.2	19.59	19	100–191	136.4	29.21
30–40	14	119–201	165.0	24.89	9	95–141	117.0	16.37
Total 0–40	103	119–226	170.8	22.76				

showed no dependence on fat content (Table 2). Analysis of 6 bovine blood samples showed a range in hemoglobin from 108 to 158 mg/g and an average of 123 mg/g. These values are close to those given by veterinary authorities (5, 6) for hemoglobin in bovine blood (Table 3). For our computation we used the reported average value of 120.3 mg/g. With the present method's recovery of 88.7%, each 107 mg hemoglobin per 100 g sample found in excess of the meat blank represents 1% added bovine blood.

Bovine blood is most commonly used for adulteration although the blood of horse, sheep, and pig could also be used. The use of blood of other species would not be practical. Table 3 shows that the average hemoglobin content for all 4 species is similar and that the computation used for added bovine blood would apply for added horse, sheep, or pig blood.

Table 2. Recovery of hemoglobin added to ground beef

Added, mg[a]	Rec., mg	Rec., %
Sample A (4.5% Fat)		
125	97	77.6
250	230	92.0
375	315	84.0
500	490	98.0
Sample B (14.5% Fat)		
125	119	95.2
250	229	91.6
375	364	97.1
500	447	89.4
Sample C (25.0% Fat)		
125	100	80.0
250	193	77.2
375	333	88.8
500	470	94.0
Av. rec., %		88.7

[a] 1, 2, 3, and 4 g bovine blood added to give 125, 250, 375, and 500 mg hemoglobin/100 g ground beef, respectively.

Confidence limits for hemoglobin content in ground beef are based on the results of 103 ground stew beef and ground beef samples. The average hemoglobin content is 171 mg/100 g with a standard deviation of 22.76. The upper limits for the 95 and 99% levels of confidence are 215 and 230 mg/100 g, respectively. Therefore, any sample with a hemoglobin content above 230 mg/100 g (i.e., 0.6% added blood) contains added blood with 99% confidence.

A ruggedness test (7) of the proposed method showed that the method is fairly insensitive to modest changes in operating procedure. The test showed only 2 areas with a large potential for error, the sample weight and the concentration of ammonium sulfate in cyanide reagent B. Experiments have shown that the ammonium sulfate solution must be between 84 and 86% to ensure complete precipitation of the hemoglobin while not precipitating the myoglobin (Fig. 2).

To calculate the errors in the determination of hemoglobin, multiple analyses of one sample and repeated analyses of 2 different samples were run by a single analyst. Also single analyses of 2 samples were run by 4 different analysts. The results gave values of 15.02 mg hemoglobin/100 g for the total standard deviation of the method. The random systematic

Table 3. Hemoglobin (mg/g) in blood of various species

Species	Range (6)	Normal (5)
Horse	110–190	124.0
Cow	80–150	120.3
Sheep	80–160	111.8
Pig	100–160	119.5

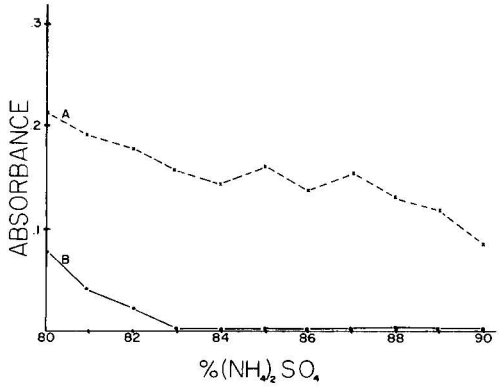


FIG. 2—Absorbance of cyanomethemoglobin and cyanometmyoglobin vs. % $(NH_4)_2SO_4$: A, cyanomethemoglobin and cyanometmyoglobin extracted from ground beef; B, cyanomethemoglobin extracted from bovine blood.

errors were calculated as 12.57 and 8.22 mg hemoglobin/100 g, respectively. The ruggedness test and the error analysis indicate that the method would give repeatable analyses under normal laboratory conditions.

Studies were done on unadulterated samples stored under various conditions. One sample stored under refrigeration at 5°C was analyzed 13 times over a period of 14 days. The values recorded were fairly constant and had a standard deviation smaller than the total standard deviation for the method. One sample was analyzed for hemoglobin, frozen for 2 weeks, and re-analyzed for hemoglobin. There was essentially no change in the total hemoglobin. These tests indicate that hemoglobin content is not affected by refrigeration or freezing of samples for reasonable periods of time.

Received February 20, 1976.

Acknowledgment

The authors wish to thank Loran A. Henderson for his help in the statistical analyses.

REFERENCES

(1) Hankin, L. (1965) *JAOAC* **48**, 1122–1126
(2) Tietz, N. W. (1970) *Fundamentals of Clinical Chemistry*, W. B. Saunders Co., Philadelphia, PA
(3) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC
(4) Salwin, H., Bloch, I. K., & Mitchell, J. J. (1955) *J. Agric. Food Chem.* **3**, 588–592
(5) Runnels, R. A., Monlux, W. S., & Monlux, A. W. (1960) *Principles of Veterinary Pathology*, The Iowa State University Press, Ames, IA, p. 372
(6) Scholm, O. W. (1965) *Veterinary Hematology*, Lea and Febiger, Philadelphia, PA, p. 550
(7) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Washington, DC, pp. 33–36

This paper was presented at the 90th Annual Meeting of the AOAC, Oct. 18–21, 1976, at Washington, DC.

VITAMINS AND OTHER NUTRIENTS

Automated Fluorometric Method for the Determination of Total Vitamin C in Food Products

RAM B. ROY, ALDO CONETTA, and JERRY SALPETER
Technicon Industrial Systems, Tarrytown, NY 10951

A specific microfluorometric method for the determination of ascorbic acid, dehydroascorbic acid, and total vitamin C in food products has been automated. The procedure developed is an adaptation of the official AOAC method (secs. 43.056–43.062), except that *N*-bromosuccinimide is used instead of Norit to oxidize vitamin C. Ascorbic acid is selectively oxidized by *N*-bromosuccinimide before other interfering substances that may be present, so this method is a highly sensitive and specific technique with extensive applicability. The proposed automated method is simple, rapid, reliable, and sufficiently sensitive to analyze as little as 2×10^{-3} to 0.1 mg ascorbic acid/ml. Analytical results obtained for ascorbic acid, dehydroascorbic acid, and total vitamin C in a wide variety of food products are reported. The analytical system developed has the capability of analyzing 50 samples/hr.

The present official final action AOAC microfluorometric method (1) for determining total vitamin C in food and pharmaceutical products is based on the method developed by Deutsch and Weeks (2). Ascorbic acid is oxidized to dehydroascorbic acid with activated charcoal (Norit). Dehydroascorbic acid is reacted with *o*-phenylenediamine (OPDA) to produce a fluorescent compound. The fluorescence intensity of this compound indicates the concentration of ascorbic acid in the sample solution. Deutsch and Weeks (2) rigorously examined the sensitivity and specificity of the OPDA method and concluded that the procedure was suitable for samples containing large amounts of reducing substances or highly colored materials.

An attempt was made to semiautomate the OPDA method. Several problems were encountered when charcoal was used manually to oxidize ascorbic acid present in the extracts from meats, dairy products, and other complex mixtures of food products. The procedure was slow and time-consuming. In most cases, final results obtained were lower in comparison with the proposed method. The analytical results were affected by the grades of the charcoal used and by the method of activation.

Since the catalytic performance of charcoal in many redox reactions depends on the presence of unsaturated sites on the carbon surfaces (3), blocking or saturating these sites will completely destroy the catalytic effect of this material.

Various chemical oxidants, such as iodine, ferricyanide, chloramine-T, 2,6-dichloroindophenol, methylene blue, and *N*-bromosuccinimide, have been reported in the literature for the smooth oxidation of ascorbic acid to dehydroascorbic acid (these reactions have formed the basis for the analysis of ascorbic acid by oxidation methods). Barakat *et al.* (4) reported that *N*-bromosuccinimide serves both as a mild (stronger oxidizing agents would bring about the complete oxidation of ascorbic acid) and selective oxidizing reagent for ascorbic acid. Evered (5) reported that this reagent is immune to reductones and reductic acids, which are generally present in fruits and vegetables. *N*-Bromosuccinimide, therefore, appears to be a useful substitute for charcoal in the OPDA method of analysis for ascorbic acid.

In this paper we describe a simplified, automated analytical procedure using *N*-bromosuccinimide in lieu of Norit as an oxidant in the OPDA method for the analysis of ascorbic acid from a wide variety of food products. The main manifold of the system developed is also applicable for the analysis of ascorbic acid by employing Norit as sample oxidant in the manual AOAC method (1). The method is simple, rapid, and accurate and eliminates those factors which are responsible for variable results observed when charcoal is used in the OPDA method.

METHOD

Apparatus

Automated analyzer.—Technicon AutoAnalyzer II continuous flow analytical system (Fig. 1) was

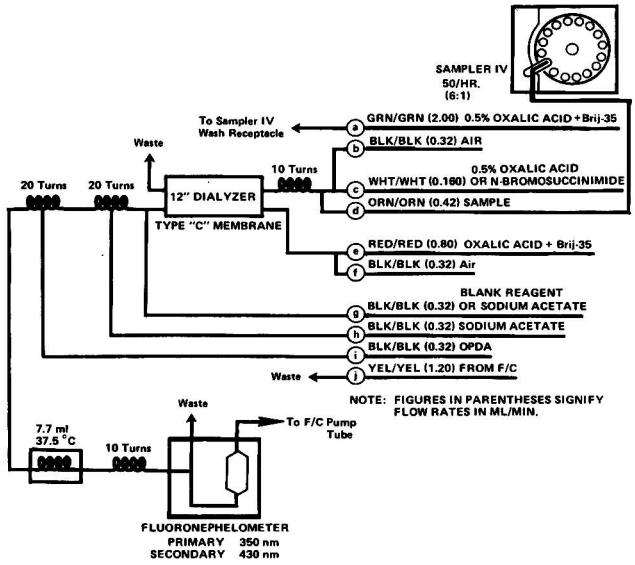


FIG. 1—Flow diagram for automated analysis of ascorbic acid, dehydroascorbic acid, and total vitamin C in food products.

constructed from the following modules: Sampler IV, proportioning pump III, heating bath with proportional controller, fluoronephelometer equipped with 365 nm excitation filter and 435 nm emission filter, and recorder.

Reagents

(a) *N-Bromosuccinimide solution.*—0.05%. Dissolve 0.5 g *N*-bromosuccinimide (analytical grade, J. T. Baker Chemical Co.) in 1 L water. Add 1 ml Brij-35 wetting reagent (Atlas Chemical Industries, Inc.) and mix. Store in brown bottle in refrigerator. Prepare fresh every 2 days (yellow indicates decomposition of reagent).

(b) *o-Phenylenediamine dihydrochloride solution.*—0.05%. Dissolve 0.5 g $C_6H_8N_2 \cdot 2HCl$ (analytical grade, J. T. Baker Chemical Co.) in 1 L water. Add 1 ml Brij-35 and mix. Store in brown bottle in refrigerator. Prepare fresh every 2 days (strong yellow indicates decomposition of reagent).

(c) *Reagent blank.*—(*1*) *Boric acid solution.*—5%. Dissolve 5.0 g boric acid (analytical grade) in 100 ml water by gently heating and stirring on hot plate. (*2*) *Sodium acetate solution.*—50%. Dissolve 50.0 g $C_2H_3NaO_2 \cdot 3H_2O$ in 100 ml water. Combine solutions 1 and 2 and check pH (8.5–9.0). If necessary, adjust pH with 50% sodium acetate solution, add few drops of Brij-35 reagent, and mix. Aqueous sodium metaborate (purified grade, Fisher Scientific Co.) solution (5%) can also be used as reagent for blank determination.

(d) *Oxalic acid solution.*—0.5%. Dissolve 5.0 g reagent grade oxalic acid in 1 L water. To 500 ml solution, add 0.5 ml Brij-35 wetting reagent and mix. Use this as reagent in sample analysis.

(e) *Activated charcoal.*—Treat 200.0 g Norit neutral (Fisher Scientific Co., carbon, decolorizing, C-170) with 500 ml 2*N* HCl solution. Heat mixture to boiling, cool, filter, and wash residue with water until free from HCl. Dry residue under water pump vacuum and finally overnight at 110–120°C. A portion of this material was activated (Roe (6)) by heating first slowly to remove water and then strongly for ca 15 min, in Erlenmeyer flask with loosely attached cover.

(f) *Ascorbic acid standard solutions.*—(*1*) *Stock solution.*—1 mg/ml. Dissolve 0.5 g ascorbic acid (reagent grade) in 500 ml 0.5% oxalic acid solution. Store in brown bottle in refrigerator. (*2*) *Working solutions.*—0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml. Pipet 2.0, 4.0, 6.0, 8.0, and 10.0 ml solution into separate 100 ml volumetric flasks. Dilute each to volume with 0.5% oxalic acid solution.

Preparation of Sample

(a) *Juices.*—Perform analysis directly on clear samples. If sample contains solid particulate, filter through Whatman No. 12 or equivalent paper.

(b) *Canned vegetables and fruits.*—Weigh suit-

able amount of sample to contain ascorbic acid in assay range and homogenize in blender in presence of 0.5% oxalic acid solution. Filter and dilute filtrate with 0.5% oxalic acid solution to known volume. Perform analysis on aliquot from final solution. Alternatively, homogenize weighed amount of sample in presence of known volume of 0.5% oxalic acid for 2–3 min. Filter and perform analysis on filtrate.

(c) *Solid samples.*—Weigh suitable amount of sample to contain ascorbic acid in assay range and add known volume of 0.5% oxalic acid solution. Homogenize 3–5 min and filter. Perform analysis on filtrate.

(d) *Meat products.*—Weigh suitable amount of sample (usually 3.0–5.0 g) and homogenize in presence of known volume of 0.5% oxalic acid solution. Filter homogenized materials through paper (use Celite as filter aid) and perform analysis on filtrate.

Methodology Notes

(a) Multiple working standards are used to establish linearity. For routine operation, a mid-scale standard is recommended for instrument calibration.

(b) Keep heating bath <40°C (preferably at 37–38°C). Higher temperature will cause *N*-bromosuccinimide to react with other, interfering materials.

(c) A 12″ dialyzer gives sufficient sensitivity. Samples with low level of ascorbic acid can be analyzed with 24″ dialyzer for greater sensitivity. Relatively clear and particulate-free samples with very low level of ascorbic acid can be analyzed without dialyzer.

(d) Oxalic acid solution (0.5%) containing Brij-35 as wetting reagent should be used as wash reagent.

(e) To wash system, place all reagent lines in water containing Brij-35 reagent. Wash system ca 25 min at end of analysis.

(f) Calculate amount of vitamin C by determining net fluorescence reading (fluorescence of sample minus blank) from chart. Amount of reduced form of vitamin (ascorbic acid) can be obtained by subtracting values of dehydroascorbic acid from values of total vitamin C.

Procedure

Before starting analysis, wash system ca 15 min with water containing Brij-35. Place all reagent delivery lines into their respective containers. After all reagents have been pumped ca 30 min, adjust baseline to read zero on chart. Place high ascorbic acid standard solution (0.1 mg/ml) on Sampler IV and adjust maximum fluorometric response to 100. Let recorder pen return to baseline before analyzing other standards and sample solutions.

To determine dehydroascorbic acid content of sample extracts, proceed as follows: Replace reagent line (c) with 0.5% oxalic acid solution containing Brij-35. Place reagent line (g) in 50% sodium acetate solution. Analyze sample, using aliquot from extract. To determine blank values, replace reagent line (g) with blank reagent solution.

To determine total vitamin C, using activated charcoal as oxidant (semiautomated AOAC method), proceed as follows: To 50.0 ml sample extract, add 2.0 g activated charcoal and shake contents vigorously 5–10 min. Filter and perform analysis on filtrate by keeping reagent lines in their respective containers as described for determination of dehydroascorbic acid.

For analysis of total vitamin C, using *N*-bromosuccinimide as oxidant, proceed as follows: Replace reagent line (c) in 0.05% *N*-bromosuccinimide solution. Determine blank value by replacing reagent line (g) with blank reagent solution. Some samples may require charcoal cleanup before analysis by this method. For such samples, proceed as follows: Filter aliquot of sample extracts through bed of charcoal supported on paper (charcoal-*N*-bromosuccinimide method). Perform analysis on filtrate.

Both automated *N*-bromosuccinimide and semiautomated AOAC methods developed are capable of analyzing 50 samples/hr.

Results

A linear relationship was observed between the absorbance and concentration of ascorbic acid in the range of concentrations studied during this investigation. The within-run precision of the automated method was determined by analyzing 30 replicates of the 0.06 mg ascorbic acid/ml standard solution (the coefficient of variation was <0.5%). The sensitivity of the method greatly increased because of the efficient oxidation of ascorbic acid by *N*-bromosuccinimide reagent. When Norit and *N*-bromosuccinimide are compared as oxidizing agents for ascorbic acid standard solution (0.1 mg/ml solution was used for 100% fluorescence reading on the chart), a difference of 20% in fluorescence (with Norit, 80%; *N*-bromosuccinimide, 100%) readings were observed. All analytical data recorded in Tables 1–7 represent the average of at least 2 replicates.

Table 1 shows a comparison of ascorbic acid

Table 1. Comparison of automated *N*-bromosuccinimide (A), semiautomated AOAC (B), manual (C), and charcoal-*N*-bromosuccinimide (D) values for analysis of cereal products for ascorbic acid (mg/oz)

Sample[a]	A	B[b]	C[c]	D[d]
1	0.9	0.7	0	—
2	19.7	21.2	19.7	—
3	22.1	24.0	20.3	—
4	21.7	25.4	20.1	—
5	20.2	22.6	20.1	—
6	20.8	22.8	21.1	—
7	20.6	21.9	22.1	—
8	20.2	22.7	20.3	—
9	22.1	24.7	20.2	—
10	20.2	22.6	20.2	—
11	35.7	23.3	21.1	24.14
12	36.3	24.0	24.7	24.99
13	36.9	24.7	23.4	24.85
14	36.9	24.7	24.2	24.99
15	36.9	24.7	22.3	25.56
16	35.9	24.0	22.9	24.85
17	39.7	28.2	26.1	29.11
18	32.5	19.0	17.8	20.80
19	43.8	31.3	28.4	31.95

[a] Samples 1-10 are corn-oat ready-to-eat cereals containing sugars and food colors. Samples 11-19 are cereals containing corn, cocoa, and sugars.
[b] 2.0 g charcoal was used for the oxidation of ascorbic acid in sample solution.
[c] Analyzed by a private laboratory.
[d] Extracts filtered through bed of charcoal supported on filter paper. Samples 1-10 were not analyzed by the automated *N*-bromosuccinimide method (D).

assays on cereal samples analyzed by the automated *N*-bromosuccinimide, semiautomated AOAC, manual, and charcoal-*N*-bromosuccinimide methods. Both the semiautomated and automated *N*-bromosuccinimide methods gave analytical results close to manual AOAC values within experimental errors. Samples 11–19 showed higher values for ascorbic acid by the automated method, when compared with the results obtained by other methods given in Table 1. Cocoa and chocolate are known to contain significant amounts of reductic acids, reductones, and alkaloids (Samples 11–19 contained these products), which might interfere with the automated method. To test this assumption, cocoa and chocolate products were analyzed by the automated method (Table 2). As is seen in Table 2, the blank reagent did not eliminate the fluorescence completely. These observations suggest that the products contained natural fluorescent compounds which may not necessarily posses ene-diol structures similar to ascorbic acid. Extracts of these samples, when filtered through charcoal, showed the complete absence of fluorescence when treated with the reagent blank.

Table 3. Effect of amounts of activated charcoal in AOAC method on determination of ascorbic acid (mg/oz)

Sample	Amount of charcoal, g[a]			
	1	2	3	4[b]
1	18.90	23.26	19.56	19.05
2	17.01	23.97	25.58	25.38
3	14.17	24.67	25.58	25.38
4	18.90	24.67	21.07	21.15
5	20.41	24.67	24.08	23.26
6	20.41	23.97	25.58	24.67
7	18.33	28.20	34.61	32.43
8	16.00	19.03	18.06	17.62
9	24.57	31.30	34.61	33.13

[a] Sample extracts were shaken vigorously in the presence of activated charcoal in the following order: 1, 1.0 g; 2, 2.0 g; 3, 3.0 g. The mixture was filtered and the filtrate was analyzed by taking an aliquot.
[b] Air was bubbled directly into the extracts for about 3 hr at room temperature. Extracts were filtered and analyzed.

Samples 11–19 were extracted with oxalic acid. The extracts were cleaned by filtration through charcoal supported on filter paper and analyzed with the *N*-bromosuccinimide method. Alternatively, the extracts were cleaned automatically by pumping a slurry of decoloring charcoal through the *N*-bromosuccinimide reagent line (c) of the manifold. (Slurry consisted of 2.0 g charcoal suspended in 2% aqueous acetic acid solution containing Brij-35. The mixture

Table 2. Percentage of fluorescence readings, as recorded on chart, from cocoa and chocolate products

Sample	With NBS[a]+OPDA	Without NBS+OPDA	With NBS	With OPDA	With reagent blank[b]
Cocoa	34	14.5	4	12	6
Chocolate	26	5	1	4	4
Mixture[c]	53	11	2	9	10

[a] NBS = *N*-bromosuccinimide.
[b] Sodium metaborate solution (5%, pH 9.2) was used.
[c] Samples were mixed in equal amounts (1.0 g of each) and assayed.

Table 4. Comparison of semiautomated AOAC and automated *N*-bromosuccinimide (NBS) methods for ascorbic acid (mg/oz) in selected food products

Food product	AOAC[a]	NBS
Canned juices		
Tomato	11.52	9.24
Orange	43.84	51.62
Grape	43.52	56.96
Vacuum-packed meats		
Pork and beef	12.82	11.70
All beef frankfurters	7.89	8.08
All beef bologna	15.75	15.11
Ham	18.20	20.00
Powdered drinks		
1	487	502
2	100	102
3	100	100
Cereals		
1	85.00	101.00
2	97.00	102.00
Canned vegetables		
Spinach	5.60	5.92
Mixed	2.80	3.24
Green beans	2.38	2.59
Beets	1.00	0.56
Sauerkraut	4.38	4.20

[a] 2.0 g charcoal was used to oxidize ascorbic acid in oxalic acid solution.

Table 5. Comparison of semiautomated AOAC and automated *N*-bromosuccinimide (NBS) methods for ascorbic acid (mg/tablet) in pharmaceutical products[a]

Sample	Amt decld	Amt found	
		AOAC	NBS
1	500	496.00	514.60
2	100	97.04	98.63
3	100	116.20	117.70
4	100	89.10	89.77
5	100	104.54	105.50
6	50	53.47	53.47
7	50	55.00	57.03
8	50	54.06	54.72

[a] Represents single and multivitamin tablets.

is stirred continuously on a magnetic stirrer.) The sample is delivered to the segmented slurry, mixed in a 5-turn coil, and dialyzed. An additional reagent pump tube, to deliver 0.025% aqueous *N*-bromosuccinimide reagent solution to the segmented dialysate, was assembled on the manifold (other arrangements on the analytical cartridge remained unchanged). This technique is suggested only when large numbers of samples containing cocoa and chocolate or both are to be analyzed routinely. This is due to the problems encountered when parts carrying carbon slurry are cleaned at the end of the analysis (most of the carbon adhering to glass and pump tube surfaces is washed out by pumping 1*N* NaOH through the system). Partially cleaned parts of the system did not affect the accuracy and the sensitivity of the automated *N*-bromosuccinimide method for the next analysis of the samples.

Table 6. Analytical results (mg/100 g) obtained by automated *N*-bromosuccinimide method for ascorbic acid, dehydroascorbic acid, and total vitamin C from selected groups of canned food products

Food product	Ascorbic acid	Dehydro-ascorbic acid	Total vitamin C
Regular Foods			
Sliced beets	3.07	0.00	3.07
Sliced carrots	2.80	0.18	2.98
Asparagus	17.30	1.92	19.22
Red cabbage	16.41	1.80	18.21
Tomato sauce	20.13	0.55	20.68
Corn	2.87	0.45	3.32
Baby Foods			
Desserts and vegetables			
Peach cobbler	25.45	6.25	31.70
Banana	33.07	5.20	38.27
Creamed corn	1.59	0.79	2.38
Creamed spinach	2.04	0.79	2.79
Peas	8.75	1.07	9.82
Peaches	23.50	2.15	25.65
Applesauce and cherry	20.57	2.15	22.72
Squash	5.40	0.00	5.40
Pears	46.00	4.00	50.00
Sweet potatoes	9.40	0.00	9.40
Banana-pineapple	53.80	3.50	57.30
Garden vegetable	8.70	0.00	8.70
Applesauce	79.00	3.42	82.42
Juices			
Orange-apple-banana	63.78	2.31	66.09
Apple-grape	40.92	1.89	42.81
Mixed fruits	79.96	2.52	82.48
Orange	62.34	1.89	64.23
Apple	71.40	2.72	74.13

Table 7. Analytical results for total vitamin C (mg/oz) obtained by automated *N*-bromosuccinimide method on canned juices and nectars

Product	Amt decld	Amt found
Juice		
Orange	120.00	154.87
Cherry	120.00	130.98
Punch	120.00	144.25
Grape	120.00	143.37
Nectar		
Apricot	80.00	78.00
Pear	80.00	81.60

Table 3 compares the ascorbic acid assays using different amounts of charcoal in the semiautomated AOAC method. Slightly lower values for ascorbic acid were obtained when 1.0 g charcoal was used. No significant differences in analytical results (except Sample 7) were observed when the amount of charcoal was increased from 2.0 to 3.0 g to oxidize ascorbic acid present in the extracts from a wide variety of food products. Moreover, air oxidation of ascorbic acid gave assay values in close agreement with samples oxidized by Norit.

A number of food samples and a few selected pharmaceuticals were analyzed both by the automated *N*-bromosuccinimide and the semiautomated AOAC procedures, respectively. Table 4 (foods) and Table 5 (pharmaceuticals) present a comparative evaluation of the analytical data obtained by these methods. In general, powdered drinks, cereals, pharmaceuticals, juices, and selected vegetables gave results by both methods which are in agreement within the limits of experimental error. It should be pointed out that, in our semiautomated AOAC method, ascorbic acid was oxidized with Norit in oxalic acid solution. The AOAC method (**43.056**) recommends oxidizing ascorbic acid in either a phosphoric-acetic-sulfuric acid mixture or a phosphoric-acetic acid solution.

In Table 6, ascorbic acid, dehydroascorbic acid, and total vitamin C analyses of a number of food products by the automated *N*-bromosuccinimide method are presented. Dehydroascorbic acid may be present in foods at the time of analysis, as shown in Table 6, or it may occur during the analysis from oxidation of reduced ascorbic acid. To obtain a reliable value for dehydroascorbic acid, it is, therefore, important to complete the analysis as soon as possible after the extraction. Although the values for total vitamin C did not change significantly by keeping the sample extracts refrigerated overnight, values for dehydroascorbic acid progressively increased.

Standard ascorbic acid solutions, when analyzed for dehydroascorbic acid, revealed that the standard (0.1 mg/ml) containing higher quantities of ascorbic acid had a smaller amount of dehydroascorbic acid in comparison to the standard (0.02 mg/ml) which contained lower quantities of ascorbic acid. This observation suggests that ascorbic acid at a higher concentration is oxidized more slowly, and, therefore, the higher potency samples should contain a smaller amount of dehydroascorbic acid.

Dehydroascorbic acid was not present in sliced beets, squash, and garden vegetables (Table 6). Red cabbage, tomato sauce, and the following baby foods (desserts), orange-apple-banana, applesauce, mixed fruits, orange, and apple, contained more ascorbic acid and less dehydroascorbic acid in comparison with the other products presented in Table 6.

Table 7 presents analytical data on several juices with high ascorbic acid content and analyzed by the automated *N*-bromosuccinimide method. The higher values obtained in comparison with the declared amount of ascorbic acid suggest that these products were generously enriched by the manufacturers.

The percentage recoveries of ascorbic acid added to the extracts from a wide variety of food products ranged from 89 to 102%.

Discussion

The proposed automated *N*-bromosuccinimide method, when comparing the chemical principles and the reaction sequences (Fig. 1), is similar to the official AOAC method. Due to the problems in on-line use of Norit with the continuous flow analytical system, ascorbic acid is oxidized to dehydroascorbic acid in the present method by *N*-bromosuccinimide reagent. Kirk and Ting (7) reported a similar automated fluorometric method, using 2,6-dichloroindophenol in lieu of Norit as the oxidant for ascorbic acid. Although the literature shows that ascorbic acid can be readily oxidized to dehydroascorbic acid by a variety of oxidizing agents, most of these oxidants bring about complete degradation of the vitamin.

Roe (6) noted that Norit serves both as an efficient clarifying agent (removes interfering materials from acid extracts of plant and animal tissue) and as an oxidant for ascorbic acid. We have observed that the oxidation of ascorbic acid by Norit in oxalic acid solution appears to depend on the amount of Norit used rather than on the time of standing. Some samples of plant origin, which contained pectins, gave abnormal results for ascorbic acid when the extracts of these samples were pretreated with Norit and analyzed by the Roe method (6).

Roe further suggested that the acid-washed

Norit should be activated first by heating gently at a low temperature to remove water and then at red-hot for about 15 min. It is known that many varieties of carbon undergo extensive changes in surface properties and reactivity after heat treatment at 400–600°C (8). Activation of acid-washed Norit at higher temperatures, according to the Roe method, is likely to produce Norit with increased efficiency and greater catalytic activity for the oxidation of ascorbic acid to dehydroascorbic acid.

We have observed that the extracts from certain groups of food products in oxalic acid solution, when treated with Norit prepared by the AOAC and Roe methods, gave slightly different assays for ascorbic acid. In general, the results obtained by using Norit (activated by Roe's method) in the semiautomated AOAC method compare more closely (within experimental limits) with those obtained by the automated *N*-bromosuccinimide method. However, where differences are observed, it could be due to either the incomplete oxidation of ascorbic acid by Norit or the decomposition of dehydroascorbic acid on a carbon surface in an acidic medium.

With respect to the selection of an extraction medium for ascorbic acid from food products, the literature indicates that oxalic acid and metaphosphoric acid either alone or in conjunction with acetic acid, sulfuric acid, or both are widely used. Some concern regarding the efficiency of metaphosphoric acid when used in varying amounts for the extraction of ascorbic acid either alone or in the presence of other acids has appeared in the literature (9).

In our procedure, oxalic acid is used both as diluent for sample and standard and extraction of ascorbic acid from a variety of food products. In most cases, there were no significant differences in analytical data obtained for ascorbic acid when the extraction procedure was carried out on a wide variety of food samples either by oxalic acid or by the AOAC-recommended extraction solutions.

Application of *N*-bromosuccinimide as a selective oxidizing agent depends on the solvent, pH of the medium, and other reaction conditions. Barakat *et al.* (4) have examined the selective oxidation of ascorbic acid with this reagent in the presence of a series of potentially interfering substances and concluded that many of these interfering materials reacted more slowly than ascorbic acid or do not react at all.

Deutsch and Weeks (2) checked the recoveries of the added ascorbic acid from various substances, using Norit as an oxidant in the OPDA method, and found that among the many substances tested, only pyruvic acid interfered. On repeating similar recovery studies, using *N*-bromosuccinimide in lieu of Norit in the OPDA method, no interferences either by pyruvic acid or by other substances (2) were observed. The major drawback in the manual OPDA method is the sensitivity of the OPDA reagent to light (develops fluorescent compounds due to the effect of light). This effect is neutralized in the automated procedures, since both the standard and samples are analyzed under similar experimental conditions.

References

(1) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, secs. 43.056–43.062
(2) Deutsch, M. J., & Weeks, C. E. (1965) *JAOAC* **48**, 1248–1256
(3) Puri, B. R. (1973) Extended Abstracts and Program, 11th Biennial Conference on Carbon, Gatlinburg, TN, June 4–8, pp. 76–77
(4) Barakat, M. Z., Abdel-Wahale, M. F. A., & El-Sadr, M. M. (1955) *Anal. Chem.* **27**, 536–540
(5) Evered, D. F. (1960) *Analyst* **85**, 515–517
(6) Roe, J. H. (1936) *J. Biol. Chem.* **116**, 609–619
(7) Kirk, J. R., & Ting, N. (1975) *J. Food Sci.* **40**, 463–466
(8) Rivin, D., & True, R. G. (1973) Extended Abstracts and Program, 11th Biennial Conference on Carbon, Gatlinburg, TN, June 4–8, pp. 80–81
(9) Loeffler, H. J., & Ponting, J. D. (1942) *Ind. Eng. Chem. Anal. Ed.* **14**, 846–849

This paper was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Received December 18, 1975.

New Method for the Determination of Free Amino Groups in Intact Pure Proteins: Relationship to Available Lysine

JAMES M. PURCELL, DANIEL J. QUIMBY, and JAMES R. CAVANAUGH
Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, PA 19118

A new rapid method for the quantitative and routine determination of free amino groups in intact pure proteins has been developed. Primary amino groups are labeled with fluorescamine and the labeled groups are detected by absorption spectroscopy in the range 375–390 nm. The amino group concentration can be determined in a few minutes without hydrolyzing the labeled protein and extracting a lysine derivative. The method was tested with the following proteins: lysozyme, α-lactalbumin, β-lactoglobulin, bovine serum albumin, ribonuclease, ribonuclease-S-peptide, and α_{s1}-casein B. Application of this method to the estimation of available lysine is discussed.

The availability of lysine for assimilation requires the presence of a primary amino group on the ε-carbon (1). If, before degradation, the ε-amino group has reacted to form any other functional group, then the lysine is unavailable. In a chemical procedure the detection of available lysine becomes the detection of the lysine ε-amino group. This group will react readily with a variety of reagents. In existing procedures for available lysine (1), lysine residues have been reacted with 1-fluoro-2,4-dinitrobenzene, trinitrobenzene-sulfonic acid, *O*-methylisourea, ethyl chloroformate, Remazol blue, and *S*-ethyl trifluorothioacetate among others. All of these methods, except the *S*-ethyl trifluorothioacetate method developed in our laboratory (2), involve complex, tedious manipulations for protein hydrolysis and extraction of labeled lysyl derivatives. With fluorescamine as the labeling reagent, these difficulties can be overcome. Fluorescamine reacts rapidly and specifically with primary amino groups to form a complex with a readily distinguishable visible absorption. A simple, routine, quantitative method for determining the labeled groups has been developed and tested with a number of pure proteins.

This research was performed under tenure of a National Research Council-Agricultural Research Service Postdoctoral Research Associateship awarded to D. J. Quimby.

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.*—Cary Model 14.

(b) *Mixer.*—Vortex type (No. 8294-F10, Arthur H. Thomas Co., Philadelphia, PA 19106), or equivalent.

(c) *pH Meter.*—Model 10 (No. 475010, Corning Glass Works, Corning, NY 14830), or equivalent, equipped with pH electrode (Arthur H. Thomas Co. No. 4094-L60), or equivalent.

(d) *Proteins.*—The following pure proteins were obtained as gifts: hen egg white lysozyme, ribonuclease, and bovine serum albumin from S. N. Timasheff; β-lactoglobulin from R. E. Townend; α-lactalbumin from M. P. Thompson; and α_{s1}-casein B from H. M. Farrell. Other proteins and peptides of the highest purity are available from commercial sources: bovine serum albumin (Miles Laboratories Inc., Kankakee, IL 60901); ribonuclease-*S*-peptide, Grade XII-PE, and lysozyme, hen egg white, Grade I (Sigma Chemical Co., St. Louis, MO 63118).

(e) *Fluorescamine solution.*—Dissolve 150 mg fluorescamine (Fluram®, Roche Diagnostics, Nutley, NJ 07110) in 50 ml dioxane (scintillation grade, Eastman Kodak Co., Rochester, NY 14650). Prepare solution in advance and let stand 24 hr at room temperature before using. Thereafter, store at 0–5°C when not in use. After 2 weeks, discard any unused portion.

Experimental

Titrate 0.15*M* boric acid (J. T. Baker Chemical Co., Phillipsburg, NJ 08865) with 0.25*M* NaOH to pH 9.5–10.0. Measure pH with glass electrode and pH meter and apply no correction factor.

Prepare aqueous protein solutions (0.4–0.8 mg protein/ml solution) in advance and store at 0–5°C until used. Dissolve water-insoluble proteins directly in buffer and dilute with water. Prepare sample and reference solutions in 100 × 13 mm culture tubes. Add 2 ml buffer to 1 ml protein solution (sample) or to 1 ml water (reference). Add 1 ml fluorescamine solution rapidly to each tube from pipet (e.g., Finn pipet) while buffered

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.

protein solution is rapidly agitated on vortex mixer. Let stand 5–10 min, and then transfer appropriate portions of sample and reference to matched pair of 1 cm cells. Measure maximum differential absorbance between sample and reference in range 375–390 nm. Use simpler differential absorbance technique rather than fluorescence, because spectroscopic sensitivity is not a problem.

Results and Discussion

The new reagent, 4-phenylspiro-[furan-2-(3H)-1′-phthalan]-3,3′-dione, is commonly referred to as fluorescamine (Fluram) and was first synthesized by Weigele *et al.* (3, 4). Fluorescamine reacts specifically with primary and secondary amino groups to yield a fluorescent and a nonfluorescent product, respectively (5, 6). Fluorescamine does not react with protonated or tertiary amino groups but is readily hydrolyzed. Fluorescamine and its hydrolysis products are not fluorescent. Recently, many applications of the specificity of fluorescamine for amino groups have been developed (7–15). Analysts who use this reagent should see the precautions mentioned in refs. 7 and 16. We have now demonstrated that this reaction can be used to measure the quantity of amino groups present in intact proteins in solutions.

Several considerations led to the method of sample preparation described above. The pH of the solutions must be high enough to allow the protein amino groups to react with fluorescamine. The pK_a values of the ε-amino groups of lysine residues fall between 9.4 and 10.6 (17). This suggests that a pH between 9 and 11 is appropriate. At a pH in this range, the protein molecules will also tend to unfold which will increase the accessibility of the amino groups to fluorescamine. Buffer is added to protein solutions to adjust the pH. The buffer should be relatively free of impurities which can react with fluorescamine. Water-insoluble proteins can be dissolved directly in buffer. Boric acid (0.15*M*) titrated with 0.25*M* NaOH fulfills these requirements.

The second consideration is that fluorescamine undergoes rapid hydrolysis. Consequently, 2 competing reactions take place when fluorescamine is added to proteins in aqueous solution: labeling and hydrolysis. The former is complete in approximately 1 sec, while the latter is complete in approximately 5 sec. To avoid adding

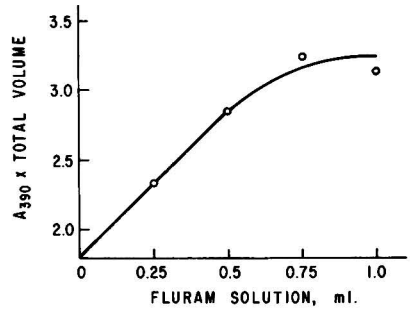


FIG. 1—Variation of A_{390} of fluorescamine-reacted β-lactoglobulin at pH 9 as function of fluorescamine solution ($7.2 \times 10^{-3}M$) added to 1 ml 0.75 mg protein/ml solution.

the fluorescamine directly, a suitable water-miscible organic solvent, such as dioxane or acetone, is used as a vehicle, and the fluorescamine solution is mixed very rapidly with the buffered protein solution.

In order to determine the amount of fluorescamine needed for complete labeling, the visible absorption of the fluorescamine complex was measured as a function of the fluorescamine solution added to solutions of α-lactalbumin and of β-lactoglobulin at several pH values. The results for β-lactoglobulin at pH 9 are shown in Fig. 1. At saturation, the molar ratio of fluorescamine to lysine is about 10:1. One ml of a $7 \times 10^{-3}M$ (~2.0 mg/ml) fluorescamine in dioxane solution is adequate for 1 ml of a (1 mg/ml) protein solution.

The method was applied to samples of α-lactalbumin and of β-lactoglobulin at constant concentration and at several pH values. For some of the samples, the pH was measured after each step of the sample preparation. The addition of fluorescamine/dioxane to a buffered protein solution increases the apparent pH by 0.5–1.0. This merely indicates a trend. A pH meter reading is not valid measurement for an aqueous organic solution. On this basis the pH of the buffer solution before sample preparation should be 9.5–10.0. With samples within this range, the results were independent of the pH value.

We readily established a linear correlation between visible absorption and amino group concentration. The absorption maximum for the labeled amino group occurs between 375 and 390 nm. Because impurities in the buffer also react with fluorescamine to give a yellow solu-

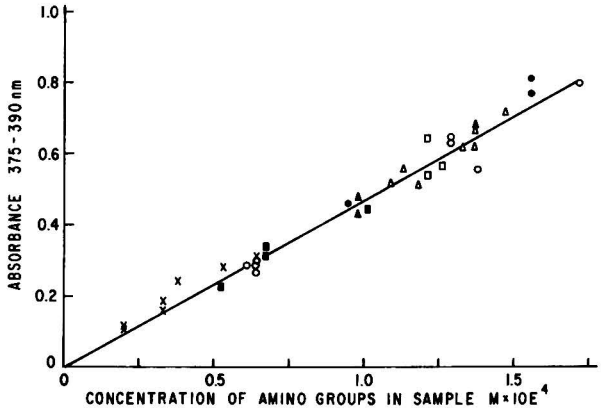


FIG. 2—Linear correlation between —NH_2 concentration and absorbance: X, ribonuclease-S-peptide; ○, α-lactalbumin; ■, lysozyme (HEW), ●, ribonuclease; ▲, α_{s1}-casein B; △, β-lactoglobulin; □, bovine serum albumin. Solid line is linear curve through origin fitted to all data obtained with these proteins in pH range 9.0–10.0.

tion, one must measure the differential absorption between the sample and a reference solution.

The method was developed and tested with a series of proteins of known amino acid sequence (18). These included hen egg white (HEW) lysozyme and 6 bovine proteins: α-lactalbumin, β-lactoglobulin, ribonuclease, ribonuclease-*S*-peptide, serum albumin, and α_{s1}-casein B. Data for these proteins were fitted to a linear curve passing through the origin as shown in Fig. 2. Thus,

$$A = \varepsilon \times C$$

where C = molarity of protein primary amino groups; A = maximum absorbance between 375 and 390 nm; and ε = molar absorptivity of labeled amino residues. The slope of the curve gave a value for $\varepsilon = 4.859 \times 10^3/M$-cm. The fit is very good; the standard error in ε for all points is 0.5%.

With ε established for labeled amino groups, one can calculate the molarity of the free amino groups in a protein from an absorbance measurement of the labeled material. Moreover, the amino group concentration can be expressed as the weight per cent of lysine in a protein as though all labeled groups were lysine residues.

$$\text{Lysine, } \% = (C \times MW \times 100)/(V \times W)$$

where C is defined above, MW = molecular weight of lysine residues, V = ratio of volume of protein solution in sample to total volume of sample, and W = concentration of protein solution in mg/ml used to make initial protein solution.

The method described above is simple, routine, and quantitative for the determination of free amino groups in intact proteins. This method can be used directly to estimate the content of available lysine. The method requires only 2 steps—labeling the protein with fluorescamine and measuring visible absorption in the range 375–390 nm. Protein hydrolysis and the extraction of a labeled lysine derivative are not necessary. The results yield a direct estimate of the unsubstituted ε-amino groups and, hence, of the available lysine of proteins. However, the α-amino group of the protein chain is also labeled, and is a minor contribution to the results obtained. How this would affect the determination of available lysine is illustrated in Table 1. The true percentage of lysine is shown in the second column, and corresponds directly to the number of lysine residues shown in column 1. The value that would be calculated by this method which would include the α-amino label is shown in the third column. The difference, of course, is related to the ratio of α- to ε-amino groups in the particular protein.

When the protein contains a substantial number of ε-amino groups per chain, the lysine determination would be excellent; for proteins or peptides with a small number of lysine residues

Table 1. Comparison of true and apparent lysine contents for several proteins

Protein	No. of lysine residues[a]	True lysine content[b]	Apparent lysine content[b,c]
Lysozyme, hen egg white	6	5.4	6.3
α-Lactalbumin	12	10.8	11.7
β-Lactoglobulin	15	10.5	11.2
Ribonuclease	10	9.4	10.3
Ribonuclease-*S*-peptide	2	10.2	15.3
Bovine serum albumin	60	11.4	11.6
α_{s1}-Casein B	14	7.8	8.4

[a] Ref. 18.
[b] Grams lysine/100 g protein, based on known amino acid sequence.
[c] Includes one α-amino group label.

per chain, for example, ribonuclease-*S*-peptide, the results would not be as good. Nevertheless, the ease and rapidity of the determination offers clear advantages. For well defined systems, that is, those for which the ratio of ε- to α-amino groups is known, appropriate correction factors can be applied to account for the α-amino label. Perhaps most important, the determination measures the content of unsubstituted lysine residues and forms the basis for the direct estimation of available lysine.

References

(1) Carpenter, K. J., & Booth, V. H. (1973) *Nutr. Abstr. Rev.* **43**, 424–451
(2) Ramirez, J. E., Cavanaugh, J. R., Schweizer, K. S., & Hoagland, P. D. (1975) *Anal. Biochem.* **63**, 130–134
(3) Weigele, M., Blount, J. F., Tengi, J. P., Czaikowski, R. C., & Leimgruber, W. (1972) *J. Am. Chem. Soc.* **94**, 4052–4054
(4) Weigele, M., De Bernardo, S. L., Tengi, J. P., & Leimgruber, W. (1972) *J. Am. Chem. Soc.* **94**, 5927–5928
(5) De Bernardo, S., Weigele, M., Toome, V., Manhart, K., & Leimgruber, W. (1974) *Arch. Biochem. Biophys.* **163**, 390–399
(6) Felix, A. M., Toome, V., De Bernardo, S., & Weigele, M. (1975) *Arch. Biochem. Biophys.* **168**, 601–608
(7) Stein, S., Bohlen, P., Stone, J., Dairman, W., & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 202–212
(8) Bohlen, P., Stein, S., Dairman, W., & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 213–220
(9) Felix, A. M., & Jimenez, M. H. (1973) *Anal. Biochem.* **52**, 377–381
(10) van Zon, A., & Beyerman, H. C. (1973) *Helv. Chim. Acta* **56**, 1729–1734
(11) Ragland, W. L., Pace, J. L., & Kemper, D. L. (1973) *J. Int. Res. Commun.* **1**, 7–8
(12) Schwabe, C. (1973) *Anal. Biochem.* **53**, 484–489
(13) Hirsch, H. E., & Parks, M. E. (1973) *J. Neurochem.* **21**, 453–456
(14) Toome, V., De Bernardo, S., Manhart, K., & Weigele, M. (1975) *Anal. Lett.* **7**, 437–440
(15) Toome, V., & Manhart, K. (1975) *Anal. Lett.* **7**, 441–443
(16) de Silva, J. A. F., & Strojny, N. (1975) *Anal. Chem.* **47**, 714–718
(17) Cohn, E. J., & Edsall, J. T. (1973) *Proteins, Amino Acids and Peptides*, Reinhold Publishing Corp., New York, NY, p. 445
(18) Dayhoff, M. O. (1972) *Atlas of Protein Sequence and Structure*, Vol. 5, National Biomedical Research Foundation, Silver Spring, MD

Received March 26, 1976.

Thin Layer Chromatographic Estimation of Available Lysine in Dried Milk Powder

SHOMPA DATTA and SALIL C. DATTA
Department of Chemistry, University of Kalyani, Kalyani, Nadia, West Bengal, India

A rapid method for the estimation of available lysine in dried milk powder has been developed. Samples were treated with 2,4,6-trinitrobenzene sulfonic acid and then hydrolyzed with 6*N* HCl. The hydrolysate was purified and subjected to ascending thin layer chromatography to isolate ε-trinitrophenyl lysine in pure form. The color was read at 346 nm in a spectrophotometer.

In any processed food or feed, available lysine content probably depends on the processing procedure. This is also true for dry milk (1–4) which is manufactured by various modern techniques. Datta (5, 6) has shown that there is a good correlation between the biologically measured protein quality and the chemically measured lysine availability for one-stomach animals. This particular amino acid plays an important role in the determination of nutritive values of food products; therefore, we thought it necessary to develop a standard procedure to estimate available lysine content in dried milk powders. Holm (7) used thin layer chromatography (TLC) and 2,4-dinitrofluorobenzene (DNFB) (8) to determine non-*N*-terminal lysine in milk. The DNFB method is tedious, so Holsinger *et al.* (9) tried to determine available lysine values in different fractions of casein by using 2,4,6-trinitrobenzene sulfonic acid (TNBS) but found anomalous results. We applied ascending TLC and TNBS to determine the available lysine content in dried milk powders.

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.*—Beckman DB-G.

(b) *Automatic sample applicator.*—Chromaplot (Burkard Ltd., Hertfordshire, England).

(c) *Chromatographic column.* — 45 × 1.5 cm Sephadex G-100.

(d) *Thin layer plates.*—Prepare silica gel layers 0.4 mm thick on 20 × 10 cm frosted glass plates.

(e) *Developing chamber.* — Approximately 35.5 × 35.5 × 10 cm covered glass chamber, lined with filter paper.

Preparation of Sample Hydrolysate

Four samples of dried milk powder, prepared by different manufacturing companies and designated A, G, L, and N were procured from the local market.

Follow method of Kakade and Liener (10) and use TNBS to prepare hydrolysates of dried milk powder and cow and buffalo milk caseins. Take 10–15 mg sample, and dilute hydrolysate to 25 ml in volumetric flask.

Purification of Prepared Hydrolysate

Freeze-dry 2 ml ether-free aqueous portion of sample hydrolysate and dissolve residue in 0.04*N* HCl. Equilibrate Sephadex G-100 column with 0.04*N* HCl and add sample solution. Elute yellow band in one portion with 0.04*N* HCl at 30 ml/hr. Collect ca 10 ml eluate. Extract collected fraction 5 or 6 times with 5 ml ethyl acetate-*n*-butanol (50+50) each time. Freeze-dry combined organic solvent extracts and dissolve residue in 2 ml ethyl acetate-*n*-butanol-glacial acetic acid (50+49+1).

Thin Layer Chromatography

Dry thin layer plates overnight at room temperature. Apply 0.01 ml sample and standard solutions (*see* below) by using Chromaplot. Air-dry spots at room temperature and then place plate in developing chamber previously saturated with *n*-butanol-glacial acetic acid-water (5+1+2). Let spots develop 2.5 hr. Air-dry plates. Yellow ε-trinitrophenyl (ε-TNP) lysine should be a single spot.

Quantitative Estimation

Carefully scrape separated ε-TNP lysine from TLC plate and add to centrifuge tube. Add 4 ml 1% sodium carbonate solution and heat tube 15–20 min in 60°C water bath. Cool to room temperature, centrifuge, and read absorbance at 346 nm against reagent blank, using Beckman DB-G spectrophotometer.

Preparation of Standard Solutions

Synthesize pure ε-TNP L-lysine according to Okuyama and Satake (11) and prepare solutions in range of 0.02–0.1 mg/ml. Plot calibration curve and use to calculate concentration of ε-TNP lysine in sample hydrolysate.

Table 1. Available lysine (g/100 g) content of dried milk powder

Sample	Total lysine[a,b]	Present method[b]	Rao *et al.* method (12)[b]
A	7.2	6.1	5.9
G	7.9	6.8	6.9
L	7.6	6.4	6.6
N	8.1	6.9	6.8
Acid-pptd cow milk casein[c]	6.5	6.3	6.2
Acid-pptd buffalo milk casein[c]	5.9	5.6	5.7

[a] Determined by Moore *et al.* method (13).
[b] Mean of triplicate results.
[c] Prepared by Hipp *et al.* method (14).

Results and Discussion

In Table 1 we have tabulated the available lysine content in dried milk powder and cow and buffalo milk caseins. The available lysine values of dried milk powder were lower than the total lysine values. Holsinger *et al.* (9) reported that the available lysine content of acid-precipitated casein was higher than the total lysine value. They believed that this discrepancy was due to the presence of hexosamine in K-casein, which yielded high apparant available lysine values. We tried to overcome this difficulty by separating ε-TNP lysine on the thin layer chromatogram and were successful. The analysis was also performed with pure D-galactosamine and D-glucosamine. The R_f values of TNP-galactosamine, TNP-glucosamine, and ε-TNP L-lysine were completely different. However, we were not able to identify TNP-glucosamine and TNP-galactosamine separately in the thin layer chromatogram of TNBS-treated milk powder. The amounts of available lysine present in acid-precipitated cow and buffalo milk casein were consistent with total lysine (Table 1).

A Sephadex G-100 column and solvent extraction techniques were used to separate ε-TNP lysine from interfering components (Figs. 1 and 2).

Two sets of recovery experiments were performed to test the specificity of the method. In one set known amounts of pure ε-TNP L-lysine were mixed with sample A, and in another set known amounts of pure L-lysine were mixed with the same sample. Reactions with TNBS were performed under the same conditions used for the sample alone. The recoveries were determined by estimating the sample hydrolysates according to our method. The mean percentage recovery of added ε-TNP L-lysine and L-lysine were 99.0 and 97.7%, respectively (Tables 2 and 3). There is a conflict in the literature (15) regarding the use of ε-TNP L-lysine and pure lysine as standards in recovery experiments, although no logical explanation has been cited. We obtained good results, so it is evident that interfering components were removed by using several purification steps.

We also determined available lysine contents of all the aforesaid samples by following the method of Rao *et al.* (12) who used DNFB. No significant difference was observed when we compared these results with those obtained by our method (Table 1). Because only 1 hr is required to hydrolyze the TNP-proteins, the present method is more rapid than the DNFB procedure.

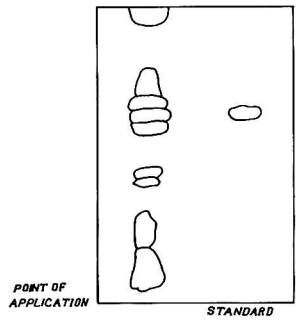


FIG. 1—Separation of ε-TNP L-lysine from the sample hydrolysate on thin layer chromatogram; *n*-butanol-glacial acetic acid-water (5+1+2).

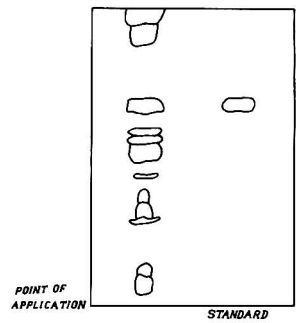


FIG. 2—Separation of ε-TNP L-lysine on thin layer chromatogram after passing the sample hydrolysate through Sephadex G-100 column and extracting with *n*-butanol-ethyl acetate (50+50); *n*-butanol-glacial acetic acid-water (5+1+2).

Table 2. Recovery of ε-TNP L-lysine added to Sample A (sample contained 183.0 μg L-lysine before fortification)

Amt added, μg	Added amt found, μg	Rec., %
20	19.8	99.0
40	39.4	98.5
60	59.5	99.2
80	79.4	99.3
	Mean	99.0

Table 3. Recovery of L-lysine added to Sample A (sample contained 183.0 μg L-lysine before fortification)

Amt added, μg	Added amt found, μg	Rec., %
10	9.7	97.0
30	29.4	98.0
50	49.1	98.2
70	68.4	97.7
	Mean	97.7

Acknowledgment

We are thankful to R. Sengupta for providing laboratory facilities.

REFERENCES

(1) Tsugo, T., Yamauchi, K., & Yoshino, U. (1961) *J. Agric. Food Chem.* **35**, 888–892

(2) Pion, R. (1961) *Annu. Biol. Anim. Biochim. Biophys.* **1**, 236–247

(3) Maruyama, Y. (1962) *Acta Paediat. Jpn.* **66**, 839–845

(4) Mauron, J., Mottu, F., Bujard, E., & Egli, R. H. (1955) *Arch. Biochem. Biophys.* **59**, 433–451

(5) Datta, S. C. (1973) Ph.D. Thesis, University of Kalyani, Kalyani, India

(6) Datta, S. C. (1976) *J. Agric. Food Chem.* **24**, 542–544

(7) Holm, H. (1971) *J. Sci. Food Agric.* **22**, 378–381

(8) Sanger, F. (1945) *Biochem. J.* **39**, 507–515

(9) Holsinger, V. H., Posati, L. P., & Pallansch, M. J. (1970) *J. Dairy Sci.* **53**, 1638–1639

(10) Kakade, M. L., & Liener, I. E. (1969) *Anal. Biochem.* **27**, 273–280

(11) Okuyama, T., & Satake, K. (1960) *J. Biochem.* **47**, 454–466

(12) Rao, S. R., Carter, F. L., & Frampton, V. L. (1963) *Anal. Biochem.* **35**, 1927–1930

(13) Moore, S., Spackman, D. H., & Stein, W. H. (1958) *Anal. Chem.* **30**, 1185–1206

(14) Hipp, N. J., Groves, M. L., Custer, J. H., & McMeekin, T. L. (1952) *J. Dairy Sci.* **35**, 272–281

(15) Hall, R. J., Trinder, N., & Givens, D. I. (1973) *Analyst* **98**, 673–686

Received May 3, 1976.

COFFEE AND TEA

High-Pressure Liquid Chromatography of Caffeine in Coffee

BRYAN L. MADISON, WILLIAM J. KOZAREK, and CECILIA P. DAMO
Procter & Gamble Co., Winton Hill Technical Center, Cincinnati, OH 45224

A new method is described for the determination of caffeine in coffee, based on high-pressure liquid chromatography. The caffeine is extracted from the sample with water and/or methylene chloride, and then separated from interfering materials by passing an aliquot of the extract through a high-pressure column containing sulfonated cation exchange resin, using 0.01*M* nitric acid as the mobile phase. An ultraviolet detector measures the absorption of the solution directly. The method is rapid and eliminates the lengthy separations common to other methods. The procedure was applied successfully to decaffeinated and non-decaffeinated green, roasted, and instant coffees. This method gives a more accurate measure of the caffeine content in decaffeinated coffee samples than the micro Bailey-Andrew and modified Levine methods, with equal or better precision. This method gives results equal to those obtained by the official methods for non-decaffeinated samples.

The need for a rapid and precise general purpose method for the determination of caffeine in coffee has been recognized. Generally accepted methods are shown in Table 1 along with an indication of the quality of results usually obtained. In addition, these methods are lengthy and complex, require large quantities of solvents, and are not general purpose. Recently, high-pressure liquid chromatographic (HPLC) methods (5–9) have demonstrated the potential of determining caffeine in analgesic drugs and aqueous beverages. This paper reports the application of an HPLC system to the separation and determination of caffeine in coffee.

METHOD

Apparatus

(a) *High-pressure liquid chromatograph.* — Laboratory-assembled from Du Pont Instruments Model 480 connected sequentially to Valco Model CV-6-HPax high-pressure injection valve, 4 mm × 30 cm stainless steel column, and Du Pont Instruments Model 835 ultraviolet monitor fitted with 254 nm photocell/filter assembly. Detector signal was plotted by Du Pont Instruments Model 846 recorder operated at 10 mv full scale.

(b) *HPLC column packing.*—Zipax® SCX (strong cation exchange resin) (Du Pont).

(c) *Mechanical shaker.*—Burrell Model 75.

Reagents

(a) *Nitric acid.*—0.01*M*. Prepare with boiled and cooled water.

(b) *Caffeine standard solution.*—2.0 mg/ml. Weigh 1 g anhydrous caffeine (Matheson Coleman & Bell No. CX 80-5013), dissolve in ca 250 ml hot water, cool, and dilute to 500 ml with water.

Preparation of Sample

(a) *Non-decaffeinated coffee.*—Accurately weigh 2 g either regular coffee or green coffee or 1 g regular instant coffee into 125 ml Erlenmeyer flask. Add ca 50 ml water, heat to boiling, and boil ≥5 min, shaking occasionally. Cool to room temperature. Filter directly into 100 ml volumetric flask. Wash filter paper thoroughly with water. Dilute to volume with water and mix.

(b) *Decaffeinated coffee.*—Accurately weigh 2 g regular coffee or 2 g green or 1 g regular instant coffee into 125 ml Erlenmeyer flask. Add 5 ml NH_4OH solution (1+2). Swirl flask until sample is wetted or dissolved (regular instant coffee) after mixing. Add 50 ml reagent grade CH_2Cl_2 from volumetric pipet, and stopper flask. Place on mechanical shaker and extract regular roasted and green coffee samples 15 min or regular instant coffee 2 min. Transfer contents to 125 ml separatory funnel and let 2 layers separate. Drain lower layer (CH_2Cl_2) through powder funnel, with

Table 1. Generally accepted methods for the determination of caffeine[a]

Method	Non-decaffeinated coffee	Decaffeinated coffee
Micro Bailey-Andrew (1)	=	++
Levine (2)	++	=
Modified Levine (3)	+	=
ISO (4)	=	=
HPLC	=	=

[a] =, Accepted as yielding correct (true) value; ++, tends to give high results; +, tends to give variable results (less precise).

stem loosely packed with cotton, and collect in 50 ml beaker. Transfer 30 ml aliquot of caffeine-containing extract to 100 ml beaker. Evaporate to dryness on steam bath with nitrogen. Add 5 ml water and warm on steam bath to dissolve caffeine. Transfer to 10 ml volumetric flask and dilute to volume with water.

Determination

Using loop injector, place 10 μl sample onto column with 0.01*M* HNO_3 eluant flowing at 4.0 ml/min. Measure peak height for quantitation and compare against standard calibration curve.

Standardization

Weigh 1.0 g regular instant decaffeinated coffee into 4 separate 100 ml volumetric flasks. Add 50 ml water and heat on hot plate to completely dissolve sample. Pipet into 3 of the volumetric flasks, respectively, 5.0, 15.0, and 25.0 ml caffeine standard solution to give 0.1, 0.3, and 0.5 μg caffeine/μl solution. Remaining solution serves as a blank. Cool, dilute to volume with water, and mix thoroughly. Inject 10 μl standard onto column as in *Determination*. Measure peak height of each standard. Plot μg caffeine in 10 μl vs. peak height to prepare calibration curve.

Calculations

(a) *Non-decaffeinated coffee samples.*—

$$\text{Caffeine, } \% = (\mu\text{g caffeine in 10 } \mu\text{l} \times 10^5\ \mu\text{l} \times 10^{-6}\ \text{g}/\mu\text{g} \times 100)/(\text{g sample weight})$$

(b) *Decaffeinated coffee samples.*—

$$\text{Caffeine, } \% = (\mu\text{g caffeine in 10 } \mu\text{l} \times 10^5\ \mu\text{l} \times 10^{-6}\ \text{g}/\mu\text{g} \times 100)/[\text{g sample weight} \times 30/50\ (\text{aliquot of } CH_2Cl_2)]$$

Results and Discussion

Reverse phase, high-pressure liquid chromatographic separations of caffeine were investigated with a μBondapak/C_{18} column and by cation exchange with a Zipax SCX column. Zipax SCX was preferable because of chromatographic time and reproducibility.

The reverse phase separations we examined used mobile phases of acetic acid-water, methanol-water, acetonitrile-water, and nitric acid-water. Dry-packed Zipax SCX columns were conditioned by pumping the mobile phase through the column for 2 hr prior to use. Predictably, the extent of separation of caffeine on these ion exchange packings was determined more by the pH of the mobile phase than by any other parameter. By decreasing the pH, the time of chromatography and resolution of the caffeine increased. The optimum pH was about 2, and the mobile phase chosen was 0.01*M* HNO_3. The flow rate was adjusted to 4 ml/min, which eluted the caffeine in less than 6 min.

Integration by peak height gave more reproducible answers than electronic integration because it is not affected by the inherent small

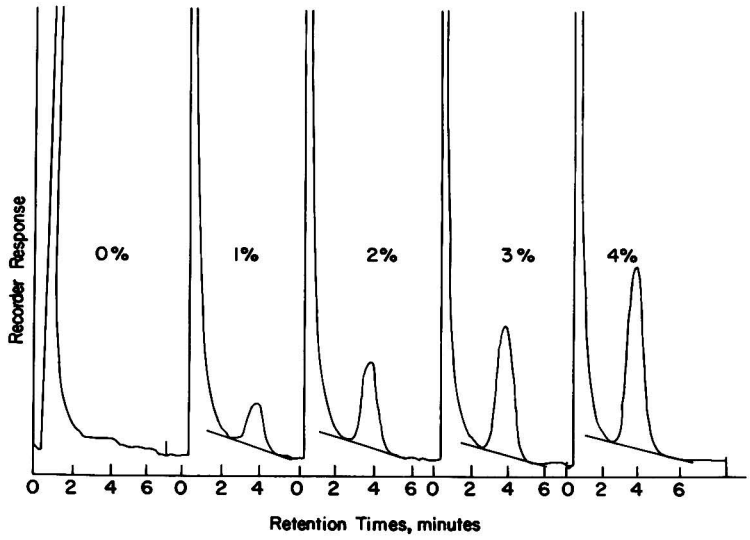


FIG. 1—Chromatograms of varying caffeine levels added to soluble decaffeinated coffee.

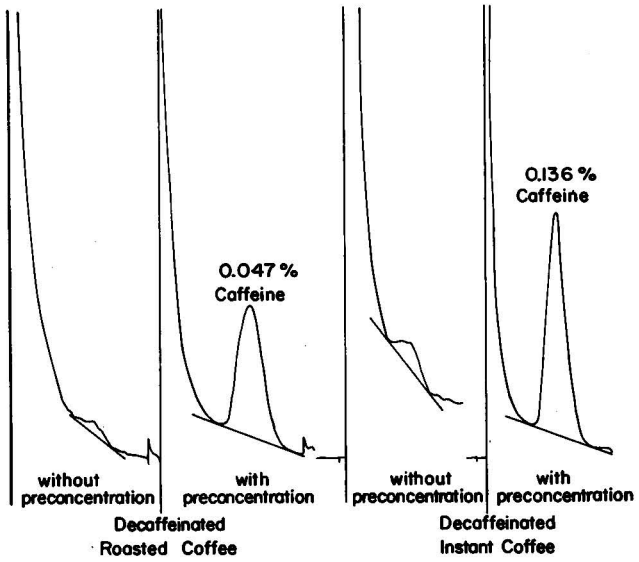


FIG. 2—Chromatograms showing effects of preconcentrating caffeine with methylene chloride.

variations in flow rate which affect the residence time in the detector and thus the area integrated.

The chromatograms obtained of caffeine-free coffee fortified with known amounts of caffeine are shown in Fig. 1. A caffeine-free coffee was used to approximate the sample matrix. Non-decaffeinated materials are easily analyzed. Caffeine is extracted from both roasted coffee and green coffee beans with boiling water, whereas instant coffee samples are dissolved directly in hot water before a small portion of the sample is introduced onto the HPLC column. Decaffeinated coffee samples require a preconcentration step before HPLC analysis. Caffeine is extracted from an NH_4OH (1+2) solution, the methylene chloride extract is evaporated to dryness, and the residue containing caffeine is dissolved in water and introduced onto the HPLC column. Typical chromatograms for decaffeinated coffee and the increased sensitivity which resulted from preconcentration are shown in Fig. 2. The peak height is more accurately measured, and the slope of the baseline decreased.

The HPLC method, the official AOAC method (1), and the proposed ISO method (4) were compared for 12 different coffee samples. The data obtained are shown in Table 2. All values obtained by HPLC agree with these accepted methods.

The coefficients of variation reported in Table 3 show that the HPLC method is more precise than the current methods now used for the determination of caffeine in coffee products.

Table 2. Comparison of 3 methods for per cent caffeine

Samples	Bailey-Andrew micro[a]	HPLC[b]	ISO[c]
Non-decaffeinated Samples			
Green coffee			
Robusta	1.91	1.91	1.95
Arabica	1.27	1.27	1.24
Roasted coffee			
Robusta		1.98	2.02
Arabica		1.30	1.31
Instant or soluble			
		3.52	3.53
		3.32	3.26
Decaffeinated Samples			
Green coffee	0.057	0.066	0.055
	0.034	0.028	0.028
Roasted coffee		0.047	0.048
		0.031	0.034
Instant coffee		0.179	0.186
		0.140	0.136

[a] Average result for 4 laboratories.
[b] Average result for 1 laboratory, 5 determinations.
[c] Average result for 8 laboratories.

Received February 9, 1976.

Table 3. Comparison of 4 methods for per cent caffeine and precision of results

Year of study	Bailey-Andrew micro		Mod. Levine		ISO		HPLC	
	Av.	Coeff. of var., %	Av.	Coeff. of var., %	Av.	Coeff. of var., %	Av.	Coeff. of var., %
Roasted and Ground Coffee								
1959	1.32	2.3						
1964			1.43	9.1				
1975					1.31	5.1	1.30	1.8
Regular Soluble Coffee								
1960	3.10	2.6						
1964			3.17	7.6				
1975					3.26	5.7	3.32	2.5
Decaffeinated Soluble Coffee								
1960	0.12	12.5						
1964			0.080	6.9				
1975					0.186	3.2	0.179	1.7

The linearity of the detector response was established from the analytical calibration curves and begins to deviate from linearity at about 10 μg caffeine injected onto the HPLC column. The lower limit of caffeine concentration that can be quantitatively analyzed by this method is 0.01% or about 0.001 μg caffeine injected.

The proposed HPLC method permits complete elimination of organic solvents in non-decaffeinated coffee samples and only a modest use of a relatively innocuous organic solvent for decaffeinated coffee samples. The method is more rapid than existing methods, 0.3 hr vs. 1.5–2.5 hr for the current or proposed methods. As many as 35 samples have been analyzed in a single working day.

References

(1) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, secs. 15.022–15.025
(2) Levine, J. (1962) *JAOAC* 45, 254–255
(3) Borker, E. (1963) *JAOAC* 46, 319–320
(4) Vitzthum, O. (1974) International Standards Organization, Document ISO/TC 34/SC 8/WG 2 (Ser 91) 233E of 1974-06-20
(5) Murgia, E., Richards, P., & Walton, H. F. (1973) *J. Chromatogr.* 87, 523–533
(6) Du Pont Liquid Chromatography Methods Bulletin (1970) 820M5, E. I. du Pont de Nemours & Co., Wilmington, DE
(7) Ascione, P. P., & Chrekian, G. P. (1975) *J. Pharm. Sci.* 64, 1029–1033
(8) Nelson, J. J. (1973) *J. Chromatogr. Sci.* 11, 28–35
(9) Smyly, D. S., Woodward, B. B., & Conrad, E. C. (1976) *JAOAC* 59, 14–19

PESTICIDE RESIDUES

Colorimetric Method for the Determination of Ethylene Dibromide Residues in Grains and Air

JOIS R. RANGASWAMY, YADATHORA N. VIJAYASHANKAR, and MYSORE MUTHU
Discipline of Infestation Control and Pesticides, Central Food Technological Research Institute, Mysore-570013, India

A method based on the hydrolytic debromination of ethylene dibromide (EDB) in the presence of an oxidizing agent has been developed. Bromine liberated from inorganic bromide is used to brominate *p*-rosaniline. The intensity of the resulting violet-red bromo compound in chloroform is measured at 580 nm. The relationship between absorbance and concentration of EDB is linear in the range of 5–60 μg. The method is very sensitive and as little as 0.50 ppm unchanged EDB residue in a 20 g sample of fumigated grain and 1 ppm EDB in a 10 ml air sample can be detected.

Ethylene dibromide (EDB) is used for the treatment of cereals in bins and bags, either alone or mixed with other fumigants. It is also used to treat fresh fruits and vegetables (1). Although it is generally believed that residues of unchanged EDB will disappear before the commodities reach the consumer, there is ample evidence from supervised trials that EDB is strongly absorbed in foods. The residues of unchanged fumigant are very slowly lost by aeration and during subsequent storage (2).

A mixture of methyl bromide (MB) and EDB was analyzed by Wade (3), using a method adapted from the catalytic combustion technique of Lubatti and Harrison (4). Recoveries of 15.3 mg EDB and 49.7 mg EDB were 97–103 and 97–100%, respectively. Dumas (5) extended the MB method of Dumas and Latimer (6) to EDB analysis. Majumder *et al.* (7) adapted the work of Hesseltine *et al.* (8) and Olomucki and Bondi (9) to determine the levels of EDB and MB in mixtures. Dumas and Bond (10) determined the unchanged EDB residues in apples fumigated with EDB by using gas chromatography with electron capture detection. The commonly employed method for residue analysis of halogenated fumigants in grains is the Stenger *et al.* iodometric method (11).

Received April 7, 1976.

Unchanged EDB residues are more significant toxicologically than residues of inorganic bromide (2). EDB is the least volatile of all common fumigants and is generally found as a residue in commodities. However, there is very little information about the extent of unchanged EDB in foods following fumigation. The available procedures either involve gas chromatography or are applicable only at high EDB concentrations. The colorimetric method described here is applicable to EDB residues in foods and air. The method is simple, quantitative, and suitable for routine analysis and monitoring of unchanged EDB residues.

METHOD

Reagents

(a) *p-Rosaniline.*—British Drug Houses grade. Prepare 0.1% solution in water.

(b) *Sulfurous acid solution.*—2.5%. Prepare by passing sulfur dioxide obtained by heating mixture of copper turnings and concentrated H_2SO_4 through 500 ml water in 1 L amber bottle. Pass gas through water until moist blue litmus paper exposed at mouth of bottle turns red. Determine concentration of sulfurous acid solution by titrating 10 ml against 0.1*N* iodine solution to faint straw yellow. Concentration of sulfurous acid solution is generally between 2.50 and 2.55%.

(c) *Potassium bromide.*—Analytical reagent grade. Prepare standard solution containing 1000 μg bromine/ml by dissolving 74.35 mg KBr in 50 ml water. Prepare working standards containing 5–50 μg bromine/ml by suitably diluting standard solution with water.

(d) *Ethylene dibromide.*—Riedel reagent grade. Prepare standards containing 5–60 μg EDB/ml by dissolving 5 mg EDB in 50 ml absolute alcohol and diluting as necessary.

(e) *Reagent A.*—Mix 10 ml 0.1% *p*-rosaniline solution with 100 ml sulfurous acid solution and shake. Within 3–5 min, the rose-red disappears, resulting in colorless solution. Age reagent $\geq$24 hr before using to prepare Reagent B, otherwise latter reagent becomes yellowish.

(f) *Reagent B.*—Add 5 ml concentrated HCl to 15 ml water in stoppered Erlenmeyer flask. While cooling flask under tap water, carefully add 10 ml concentrated H_2SO_4. After thorough cooling, add 10 ml Reagent A to this mixture. Shake well and refrigerate until use.

Conversion to Inorganic Bromide

Pipet 1 ml EDB standard solution into glass-stoppered 40 ml test tube containing mixture of 0.5 ml 100% KOH solution by weight and 0.4 ml 10% potassium chromate solution. Mix contents of tube well, stopper, and let stand 1 hr at room temperature to complete conversion of EDB to KBr. Then evaporate alcohol on wire gauze kept heated over flame. Dissolve residue in 1 ml water and use for color development. Similarly, analyze blank of 1 ml ethanol.

Preparation of Standard Curve

To 1 ml aqueous solution above, add in order and shake after each addition: 1 ml water, 0.4–0.5 ml concentrated HCl (when solution becomes orange red), 4 ml reagent B, and 0.2 ml 10% potassium chromate solution (when solution becomes reddish brown or dark brown, depending on bromide concentration). Shake well 30 sec, add 6 ml $CHCl_3$, and shake well 1–2 min to extract violet-red color with absorption maximum at 580 nm (Fig. 1) into $CHCl_3$. Transfer contents to 250 ml separatory funnel, mix, and let layers separate. Drain lower $CHCl_3$ layer into cell of spectrophotometer and read absorbance at 580 nm against blank similarly prepared within 5 min. Plot of concentration of EDB vs. absorbance will yield straight line.

Determination

Mix 1 ml concentrated solution containing ca 100–1000 μg EDB with 0.5 ml 100% KOH solution, 0.4 ml 10% potassium chromate, and 1.5 ml ethanol. Reflux mixture 3 hr on boiling water bath under water-cooled reflux condenser. Evaporate ethanol on heated wire gauze. Dissolve residue in known amount of water and use 1 ml solution for color development as in preparation of standard curve. Read color against blank similarly prepared. If the violet-red $CHCl_3$ extract is slightly yellow, add 2–3 ml water and shake to remove yellow color.

Determination in Air

Place 0.5 ml 100% KOH solution, 0.4 ml 10% potassium chromate solution, and 1.5 ml ethanol in two-necked, B24, 250 ml standard joint flask. Close one mouth of flask with B24 standard joint with bent tube through center. Fix rubber septum to outer end of tube; keep inner end of tube just under surface of mixture. Close other mouth of flask with hollow B24 standard joint to which one-way tap is fused for displacement of air. Cool flask with crushed ice. Open tap and bubble 250 ml air containing 15–200 μg EDB taken from reservoir containing EDB at saturation through rubber septum with help of syringe. Close tap and let flask stand overnight. Next day reflux mixture 3 hr on water bath, using water-cooled condenser. Remove condenser and evaporate ethanol as before. Dissolve residue in known volume of water and use 1 ml aliquot for color development. Read color against blank similarly prepared. If violet-red $CHCl_3$ extract is slightly yellow, add 2–3 ml water and shake to remove yellow color.

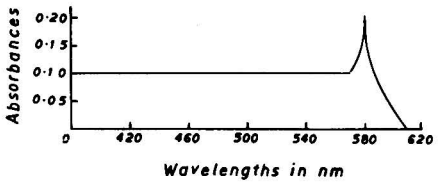


FIG. 1—Absorption maximum of bromorosaniline.

Recovery from Crops

Fortify 20 g samples of paddy, wheat, sorghum, and green gram with 40–250 μg EDB/ml as ethanolic solution in 250 ml flask. Similarly analyze blank crop by adding 1 ml ethanol. Let EDB absorb into grains and let ethanol evaporate slowly. Add 20 ml ethanol, stopper flask, and let stand overnight to dissolve EDB from grains completely in ethanol. Next day, filter and wash grains and flask with 2–3 ml ethanol, and filter. Mix alcoholic filtrates with 0.5 ml 100% KOH solution and reflux 2 hr on water bath. Remove condenser and evaporate nearly ¾ ethanol on water bath. Add 0.4 ml 10% potassium chromate solution and reflux again 1 hr. Detach reflux condenser and evaporate remaining ethanol as before. Dissolve residue in known volume of water and use 1 ml aliquot for color development. Treat crop control samples similarly. Read color against blank and subtract control reading from reading of fortified samples. If violet-red $CHCl_3$ extract is slightly yellow and turbid due to dispersed water globules, add 2–3 ml water, shake, and filter quickly.

Residue Analysis of Fumigated Grains

Subsamples were drawn from main bulks of grain (wheat, paddy, sorghum, and green gram) fumigated with 48 mg EDB/L for 7 days. The grain was then aerated in the sun for 7 days by spreading in an open yard. Residue analysis was

Table 1. Absorbance at 580 nm of bromine equivalents[a] of EDB and KBr

Bromine derived, μg	EDB	KBr
5	0.03	0.03
10	0.06	0.06
15	0.081	0.076
20	0.12	0.115
25	0.137	0.143
30	0.161	0.155
35	0.187	0.194
40	0.25	0.24
45	0.237	0.244
50	0.31	0.30

[a] Bromine equivalents: 1.487 μg KBr = 1.175 μg EDB = 1 μg bromine.

Table 2. Recovery experiments of EDB from concentrated alcoholic solution

EDB used, μg	EDB found,[a] μg	Rec., %
236.0	232.0± 4.00	98
354.0	348.0± 9.80	98
472.0	457.1±12.00	97
590.0	588.8± 5.59	98
708.0	708.0± 6.07	100
826.0	824.8± 3.36	99
944.0	937.6± 7.93	99
1062.0	1062.0± 0.00	100

[a] Average ± std dev. of 6 analyses.

done both before and after aeration by soaking 20 g sample in 20 ml ethanol as described under recovery experiments.

Results and Discussion

Hesseltine *et al.* (8) have reported that only 50% EDB converted to inorganic bromide when hydrolyzed with 5% alcoholic KOH, while Kennett (12) reported hydrolysis of only 1 mole bromine from 1 mole EDB hydrolyzed with 4% alcoholic NaOH for 15 min. Sinclair and Crandall (13) extended the MB method of Stenger *et al.* (11) by determining the conditions necessary for ethanolamine hydrolysis of EDB. A 30 min heating at 90°C was adequate for complete conversion to inorganic bromide. After many trials, we found that EDB is converted 100% to inorganic bromide when hydrolyzed with a mixture of 0.5 ml 100% KOH solution by weight and 0.4 ml 10% potassium chromate solution.

To check the 100% conversion of EDB to inorganic bromide under the conditions of hydrolysis employed, parallel experiments were performed with 1 ml standard solution containing 5–50 μg bromine equivalent of potassium bromide for color development. Results of analysis of bromine equivalents of EDB and KBr are shown in Table 1. Absorbances in Table 1 prove that under the conditions of hydrolysis employed, 100% conversion of EDB to inorganic bromide takes place in the range of 5–50 μg bromine equivalents of EDB. The conversion was inconsistent in the absence of potassium chromate solution.

The linear relationship between absorbance at 580 nm and the concentration of EDB is valid up to 60 μg/6 ml $CHCl_3$. Beyond this level the relationship is not linear and inconsistent results are obtained. To check the conversion at 1 hr at room temperature, experiments were done by refluxing the mixture for 3 hr on a water bath. No difference in absorbance was noticed.

To verify the applicability of the method to higher EDB residues, recovery experiments were performed on concentrated ethanolic solutions of EDB at 100–1000 μg. The residue remaining after evaporation of ethanol was dissolved in a

Table 3. Recovery of EDB from air samples

EDB added, μg	EDB found,[a] μg	Rec., %
15.40	15.10±0.60	98
25.00	23.86±0.38	99
50.00	48.64±0.74	97
60.00	60.00±0.00	100
77.00	75.50±3.45	97
100.00	99.00±2.40	99
107.80	104.80±0.88	97
200.00	190.88±3.04	95

[a] Average ± std dev. of 6 analyses.

Table 4. Recovery of EDB from fortified grains

EDB added, μg	EDB found,[a] μg	Rec., %
Wheat		
134.0	133.20±0.80	99
224.0	222.00±1.40	99
Sorghum		
51.0	50.00±0.00	98
102.0	97.50±0.00	95
Paddy		
56.0	55.60±0.12	99
70.0	66.00±0.65	94
Green gram		
42.0	39.75±0.25	99
84.0	81.00±0.00	96

[a] Average ± std dev. of 6 analyses.

Table 5. Residue analysis (ppm) of grains fumigated at 48 mg EDB/L[a]

Stage	Wheat	Sorghum	Paddy	Green gram
Before aeration	12.29±1.32	5.68±0.02	6.32±0.12	5.25±0.00
After aeration	4.27±0.00	1.87±0.00	3.15±0.00	1.50±0.00
After milling into flour	ND	ND	NA	NA

[a] Average ± std dev. of 6 analyses. ND = not detected; NA = not analyzed.

larger volume of water so that the concentration of bromide was within Beer's law limit (*see* Table 2). Values indicate that the method is applicable for determination of higher levels of EDB, provided the strength of the solution of the residue is within the linear relationship limit. Average recoveries varied from 97 to 100%.

For determination of EDB concentration in air, various sampling methods have been reported. Kennett (12) took air samples in 2 L evacuated flasks and ethanol was added to dissolve the gas. The solution was transferred and refluxed with NaOH for 15 min. After acidification, bromide was determined by the Volhard method. Berck (14) developed an analysis suitable for measuring EDB in EDB-MB mixtures. Samples were collected in evacuated bottles containing an ethanolamine-*n*-propanol mixture. EDB was hydrolyzed 3 hr at 100°C. In the present study, neither the evacuation of the flask nor ethanolamine is necessary. EDB airborne vapor can be bubbled through the mixture of reagents used (*see* Table 3). The result indicates that the sensitivity of the method is quite high. It is applicable for the determination of EDB in air as low as 60 μg/L, which is far less than the permissible limit of 190 μg/L (25 ppm).

Recovery experiments (Table 4) and residue analysis (Table 5) of EDB in grains such as paddy, sorghum, and green gram did not present any difficulty. For wheat, an ethanol- and chloroform-soluble material interfered during the extraction and created a viscous chloroform extract. To overcome this, 1 ml solution of the residue was mixed with 5 ml water, and extracted twice with 20 ml chloroform. The lower chloroform layer was discarded and the aqueous layer was used for color development. Although Mapes and Shrader (15) and Conroy *et al.* (16) have described various complicated procedures for eluting EDB from fumigated grain, we found that EDB can be completely eluted by soaking the grain in ethanol overnight. The method is applicable to as little as 0.5 ppm EDB compared with the permissible limit of 20 ppm in cereals (2).

Acknowledgments

The authors thank B. L. Amla for kind encouragement and S. K. Majumder for a critical appraisal of the problem. The technical help of K. P. Kashi is gratefully acknowledged.

REFERENCES

(1) Monro, H. A. U. (1969) *Manual of Fumigation for Insect Control,* FAO Agricultural Studies No. 79, p. 381
(2) WHO (1972) *1971 Evaluation of Some Pesticide Residues in Food,* WHO Pesticide Series No. 1, p. 345
(3) Wade, P. (1952) *J. Sci. Food Agric.* 3, 390–393
(4) Lubatti, O. F., & Harrison, A. (1944) *J. Soc. Chem. Ind.* 63, 140–144
(5) Dumas, T. (1962) *J. Agric. Food Chem.* 10, 476–477
(6) Dumas, T., & Latimer, R. A. (1962) *J. Agric. Food Chem.* 10, 276–279
(7) Majumder, S. K., Muthu, M., & Narasimhan, K. S. (1963) *Food Technol.* 17, 108–111
(8) Hesseltine, H. K., Hamilton, G. A., & Waller, J. B. (1951) *Pest Infestation Research,* Her Majesty's Stationery Office, London, p. 26
(9) Olomucki, E., & Bondi, A. (1955) *J. Sci. Food Agric.* 6, 592–600
(10) Dumas, T., & Bond, E. J. (1975) *J. Agric. Food Chem.* 23, 95–98
(11) Stenger, V. A., Shrader, S. A., & Beshgetoor, A. W. (1939) *Ind. Eng. Chem. Anal. Ed.* 11, 121–124
(12) Kennett, B. H. (1954) *J. Agric. Food Chem.* 2, 691–692
(13) Sinclair, W. B., & Crandall, P. R. (1952) *J. Econ. Entomol.* 45, 80–82
(14) Berck, B. (1961) cited in Malone, B. (1971) *Residue Rev.* 38, 21–80
(15) Mapes, D. A., & Shrader, S. A. (1957) *JAOAC* 40, 180–185
(16) Conroy, H. W., Munsey, V. E., & Ramsey, L. L. (1957) *JAOAC* 40, 185–189

Reference Raman Spectra of Eleven Miscellaneous Pesticides

MARK L. NICHOLAS, DAVID L. POWELL, THEODORE R. WILLIAMS, ROBERT Q. THOMPSON, and NELSON H. OLIVER
Department of Chemistry, College of Wooster, Wooster, OH 44691

The Raman spectra of ICP (propham), CIPC (chlorpropham), carbaryl, EPTC (ethyl *N,N*-dipropyl (3-chlorophenyl) carbamate), pebulate, CDEC (2-chloroallyl diethyldithiocarbamate), thiram, maneb, zineb, ferbam, and nicotine have been recorded. These spectra are presented, along with tables giving values for the frequencies.

We have recently published and discussed some possible analytical applications of the Raman spectra of 21 different chlorine-containing or phosphorus-containing pesticides (1, 2). In this paper we present Raman spectra for 11 more compounds now being used as pesticides.

The first group, which consists of carbamates or closely related compounds, covers IPC (isopropyl *N*-phenylcarbamate, propham), CIPC (isopropyl *N* - (3 - chlorophenyl) carbamate, chlorpropham), carbaryl, EPTC (ethyl *N,N*-dipropylthiolcarbamate), pebulate, CDEC (2-chloroallyl diethyldithiocarbamate), and thiram. Compounds in the second group, maneb, zineb, and ferbam, all contain a metal. Finally, nicotine stands alone in an abbreviated third group. The names employed are those used by Frear (3).

Experimental

The compounds were obtained from Chem Service, Inc., West Chester, PA 19380. They were sublimed or distilled under vacuum and sealed in melting point capillaries. The Raman spectra were obtained as described previously (1).

Results and Discussion

Tabulated frequencies and spectra for the carbamates and related carbamate-like compounds are given in Table 1 and Figs. 1–7. Frequencies and spectra for the metal-containing compounds and for nicotine are given in Table 2 and Figs. 8–11.

Obviously, each of these spectra is unique for its particular compound. Certain prominent features will be pointed out for each of the compounds. References to general correlations were given in ref. 1.

Of the first 7 compounds, IPC, CIPC, and carbaryl, the 3 compounds that possess N—H linkages, are the only ones to show activity above 3110 cm^{-1}. In these compounds, any bands primarily associated with the carbonyl region of the molecules (near 1700 cm^{-1}) are overshadowed by bands from the benzene or naphthalene ring systems. Their carbonyl bands do agree reasonably well, however, with the range found many years ago by Kohlrausch and Pongratz (4) for carbamates of the type $H_2N(=O)—OR$, i.e., 1688–1694 cm^{-1}. CIPC shows more activity than IPC in the low frequency region because of the chlorine substituent on the benzene ring in CIPC.

EPTC, pebulate, and CDEC all show bands in the C—H stretching region near 3000 cm^{-1} which are 2 or 3 times as intense as the bands in the rest of the spectra. The C=O stretching frequencies for EPTC and pebulate (at about 1640 cm^{-1}) appear more plainly than in the first 3 compounds considered. Similarly, the C=C linkage in CDEC appears very plainly at 1625 cm^{-1}, and the ethylenic CH gives rise to a peak at 3104 cm^{-1}.

In CDEC and thiram, it is tempting to assign some of the stronger bands to the C=S stretches. However, the difficulties when the carbon atom of the thiocarbonyl group is attached to a nitrogen atom often mentioned earlier (*see*, for example, the work of Rao and Venkatarazhavan (5)) make this unwise because these stretches are just too highly mixed with other modes in the compounds.

The 3 metal-containing pesticides present spectra very different from any we have considered previously. In particular, maneb and zineb show low frequency peaks which are several times more intense than any others in their spectra. Such intense peaks would offer unusual opportunities for the analyst; however, they disappear in solution, showing that they are lattice modes. Parenthetically, the almost exact correspondence between the maneb and zineb spectra,

Received June 1, 1976.

Table 1. Raman frequencies of carbamates and related compounds[a]

IPC	CIPC	Carbaryl	EPTC	Pebulate	CDEC	Thiram
	3362 vw					
3317 w		3313 vw				
	3296 w, br	3306 vw				
		3266 vw, br				
	3193 vw, br					
	3118 vw, br					
					3104 w	
3059 s	3059 m	3065 s				
	3034 vw, br				3042 w, sh, br	
		3012 w, br				
2980 mw	2977 m, br					
					2972 s	
			2961 w, sh, br	2961 w, sh		2958 w
		2940 w, br				
2936 m	2935 s					
						2930 w
			2928 vs	2933 s	2929 vs	
2918 w, sh	2916 m					
	2898 vw, br				2904 w, sh, br	
2875 w	2873 w		2869 vs	2872 s	2866 s	
						2850 w
		2811 w, br				
						2785 w, br
	2764 vw, br					
	2729 vw, br		2722 w, br	2733 m	2732 vw, br	
1696 w, br		1705 w				
	1689 m					
		1669 vw, br				
			1641 w, br	1644 s		
					1625 s	
1600 s	1594 vs	1593 w, sh, br				
		1576 s				
		1547 vw, br				
1535 w	1538 m					
1499 vw, br		1503 vw, br				
1486 vw, br						
	1478 w				1482 m	
1463 vw, br	1465 vw, br					
		1459 w, sh				1458 m, br
1448 w, br	1446 w, br		1442 w, br	1446 s	1446 m	
		1432 s				
	1427 vw, br					
		1413 vw			1410 mw	
	1401 vw, br		1402 ms	1402 m		
						1394 s
	1385 vw, br					
		1376 vs		1375 w	1370 mw	1370 s
1351 vw, br	1350 vw, sh		1348 w, br		1350 mw	
1326 vw, br	1329 vw, sh	1328 vw		1331 vw		
1310 vw, sh	1303 s					
1298 vw			1290 vw, br	1299 w, br	1294 vw, br	
	1275 m					
		1265 w	1265 w, br		1264 m	
	1251 s	1252 w, br				
1242 m						
	1228 m					1232 w
		1223 vw, br	1216 w, br	1225 w, br	1220 vw	
1207 vw, br						
		1197 vw, br		1193 vw	1200 w	
		1184 vw, br				
1172 mw	1168 vw, br	1166 w, br				
1155 w						
		1148 vw				1144 m, br
1139 vw	1142 w	1138 w			1139 w	
		1112 w	1115 w	1113 mw	1114 vw, br	
1104 w	1106 mw		1099 w, sh			
1083 vw, br				1092 w		1084 w, br
	1074 w			1075 w	1072 m	
1065 vw		1064 m				

(Continued)

Table 1. *(Continued)*

IPC	CIPC	Carbaryl	EPTC	Pebulate	CDEC	Thiram
1050 vw			1051 mw	1051 w		
		1034 w				1036 w, br
1024 w			1030 mw	1027 w		
		1013 w			1008 s	
994 vs	994 vs					
				984 w	980 m	
			966 vw, br			971 s
				959 w		955 w, sh
		951 vw, br	948 vw, br			
	935 w	937 m		938 w		
925 mw	921 w					
					912 mw	
903 vw	897 w	902 vw, br		897 m		
	887 w, br		888 w		886 vw, br	
874 m		874 w		881 m		
		861 vw, sh	866 vw, br		857 vw, br	
						847 m
			843 w			
834 vw, br				839 w, br	827 vw	
	824 m					
796 vw				804 w		
			783 vw, br	781 mw		
767 w	766 w, br				769 w, sh, br	
745 vw	755 w, br		748 w		749 mw	
		728 vs		736 m		
			709 w	702 w		
	689 w, br				681 mw	
			668 m			
663 vw				659 s		
	634 vw	623 vw, br				
					625 m	
614 w						
600 w	602 w					
					588 vw	
	568 w					
					560 s	558 vs
		536 s				
			523 vw, br			
	516 vw				517 w	
509 w		507 s				
			489 m	488 m	489 vw	
473 vw, br	472 w					
		455 m				
						439 ms
					428 s	
413 w	419 w			425 w, br		
		417 vw				
	401 vw, sh		406 vw, br			
	390 w			392 w, br	397 mw	393 vs
365 vw	371 w	370 mw	365 vw, br			
					360 w	357 m
				354 mw		
			316 vw, br		322 vs	
						313 ms
	296 w	299 vw			298 m	
		276 w				
256 w	259 w		266 ms			262 vw, br
		247 vw, br		253 s		
	232 vw, br					
	222 vw, br				218 w, br	
205 vw				206 w, sh		
	194 s					
						173 ms
		162 w				
		103 w				104 w
						67 vs

[a] The following abbreviations have been used: s = strong; m = medium; w = weak; br = broad; v = very; sh = shoulder.

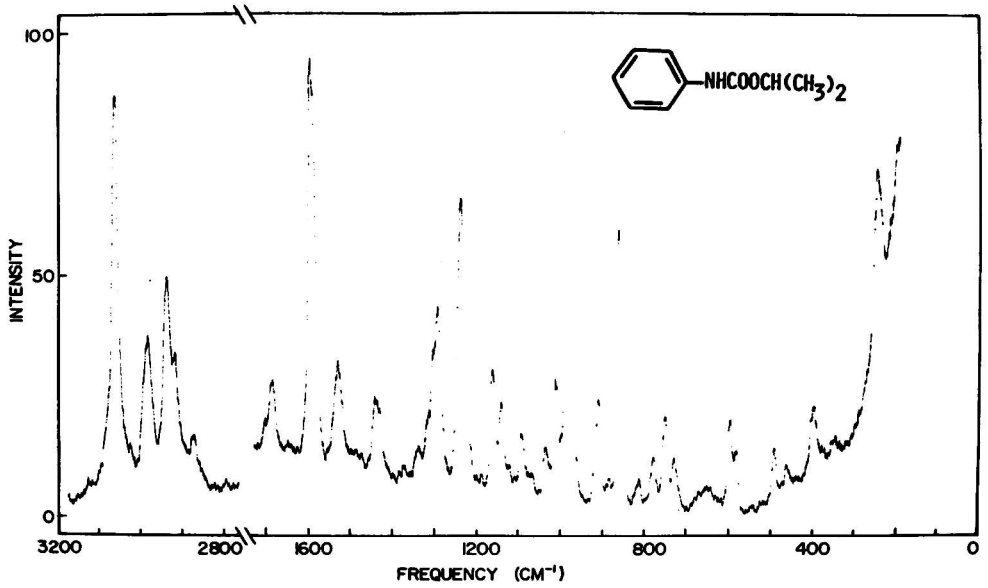


FIG. 1—Raman spectrum of IPC.

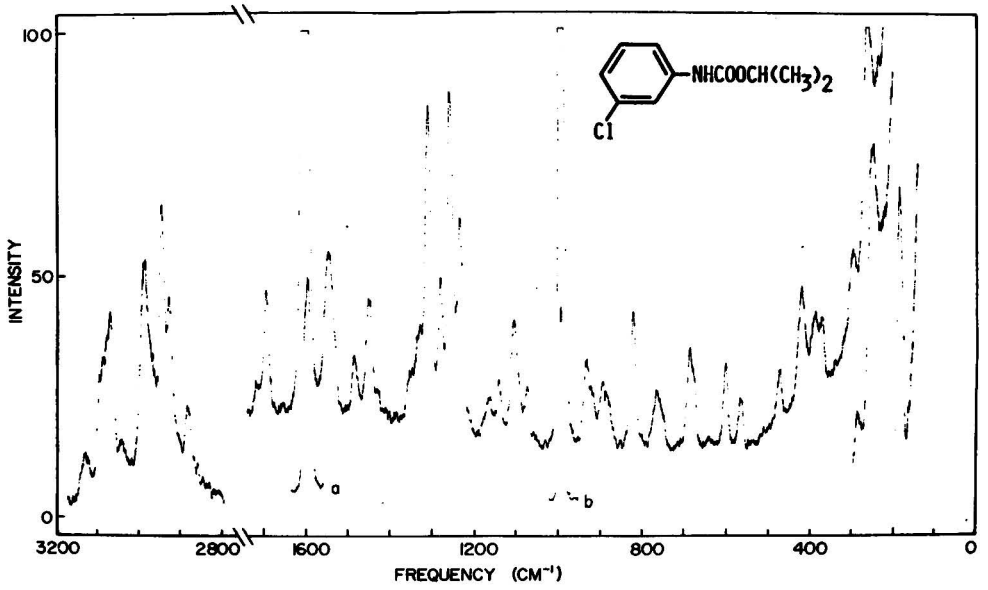


FIG. 2—Raman spectrum of CIPC. a, sensitivity ~0.33×; b, sensitivity ~0.17×.

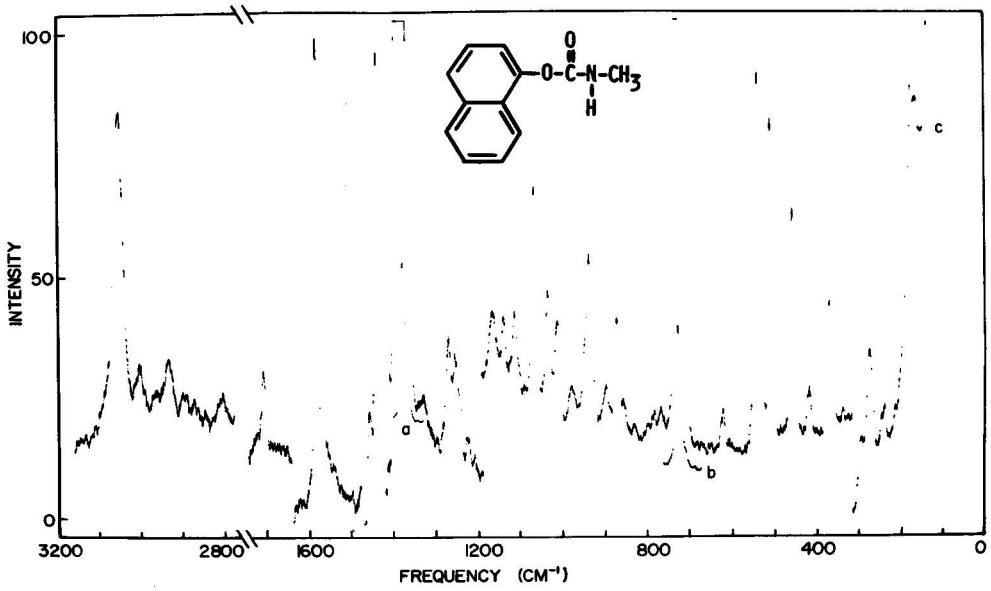


FIG. 3—Raman spectrum of carbonyl. a, sensitivity ~0.07×; b, sensitivity ~0.20×; c, sensitivity ~0.48×.

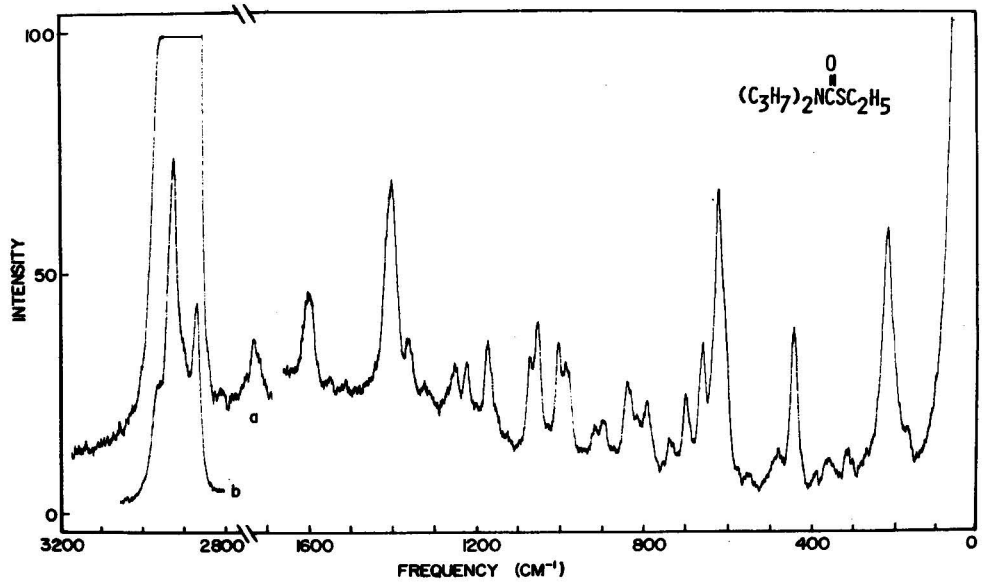


FIG. 4—Raman spectrum of EPTC. a, sensitivity ~1.6×; b, sensitivity ~0.5×.

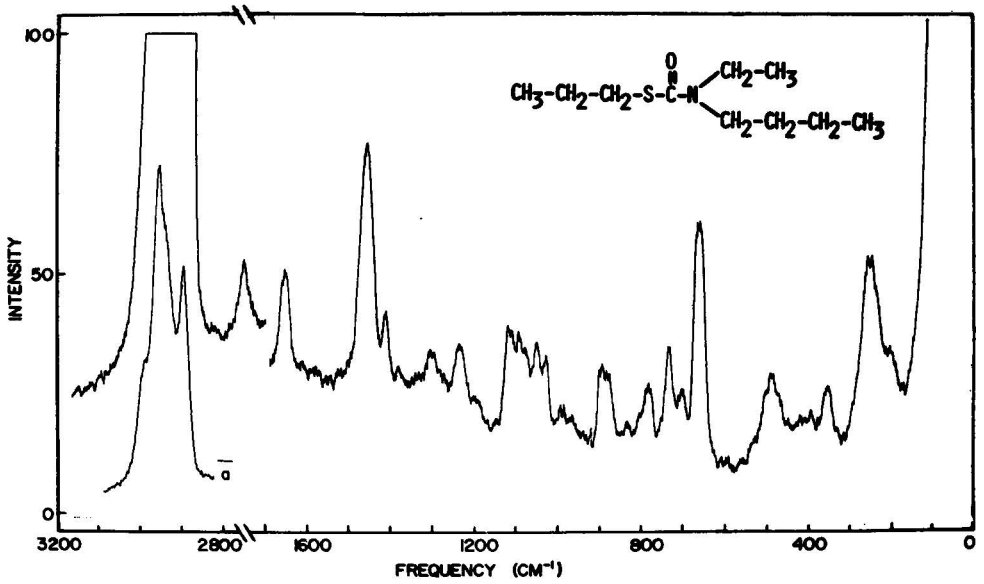


FIG. 5—Raman spectrum of pebulate. a, sensitivity ~0.33×.

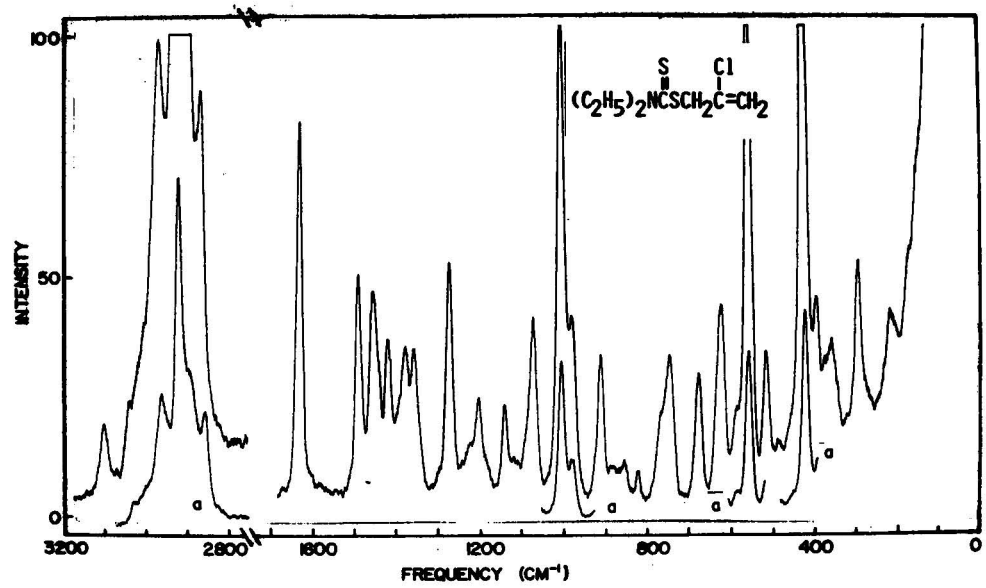


FIG. 6—Raman spectrum of CDEC. a, sensitivity ~0.33×.

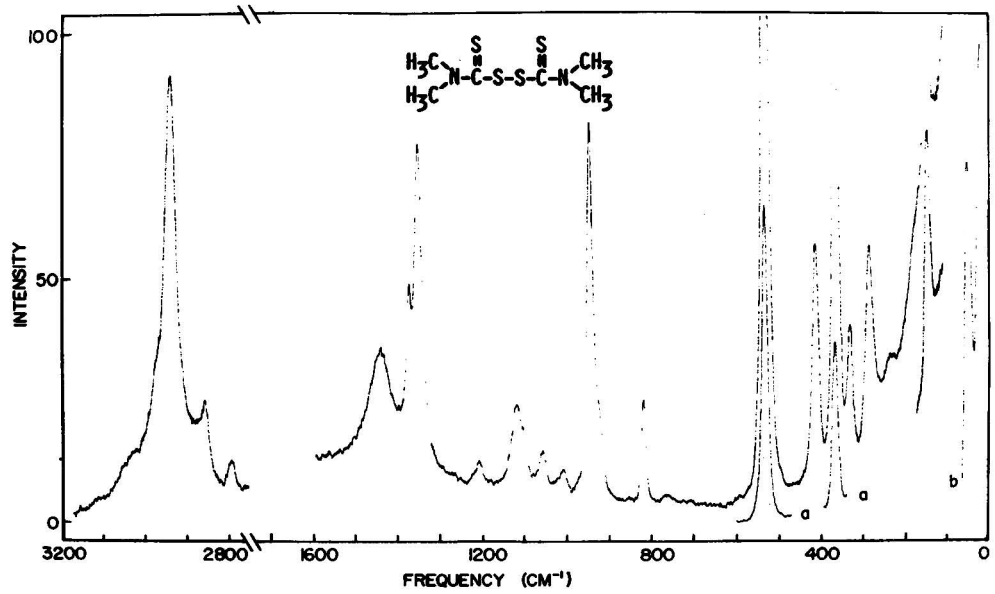


FIG. 7—Raman spectrum of thiram. a, sensitivity ~0.27×; b, sensitivity ~0.10×.

Table 2. Raman frequencies of metal-containing pesticides and of nicotine[a]

Maneb	Zineb	Ferbam	Nicotine	Maneb	Zineb	Ferbam	Nicotine
3284 w, br	3279 w, br			1048 w	1049 vw, br		
3241 w, br	3237 w, br						1038 vs
			3168 vw				1023 s, sh
			3144 vw			1099 vw, br	
			3048 s	975 w, br	976 vw, br	972 m	
		3012 w, br				958 m	
2960 w, br	2962 mw, br	2952 vw, sh	2962 w, sh	918 s	921 ms		930 s
			2939 s				911 w, sh
2928 vw, br	2930 mw, br			869 w, br		878 vw, br	
		2922 vs		816 vw, br			812 m, br
			2909 w, sh				777 w, sh
2890 mw	2892 m						716 w
			2870 mw	659 vw, br			661 w, br
		2856 w					614 m
			2825 w, sh			564 vs	568 m
		2795 w		505 s	505 ms		520 w
			2775 ms	469 vs	470 s		
			2664 w, br	433 s	434 ms	432 s	
			1584 s				402 m
		1508 m				365 w	
1479 w	1483 w, br						348 m
1461 vw, br	1462 vw, br		1471 w				297 s
		1440 w	1449 m			285 vw, br	
			1411 w, sh				254 w, sh
		1385 vs		243 s	243 w, sh		
1374 vw, br				217 vs	218 vs		
			1354 mw			216 w	
			1341 mw	184 w	185 w		
1306 w	1309 w, br		1309 m			162 ms	
		1239 w	1237 m	151 vs	150 vs		
1207 m	1209 mw		1212 m	80 vs	82 s		
			1181 m			78 vw	
		1140 m		47 s	50 s		
			1101 vw, sh	41 m	42 m		
			1092 mw				

[a] For abbreviations, see Table 1.

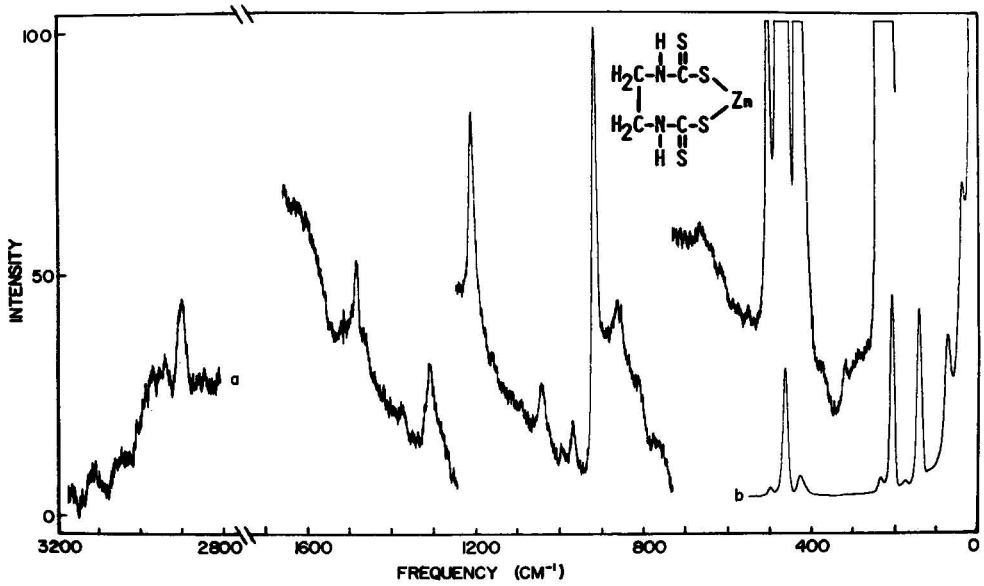


FIG. 8—Raman spectrum of maneb. a, sensitivity ~0.83×; b, sensitivity ~0.025×.

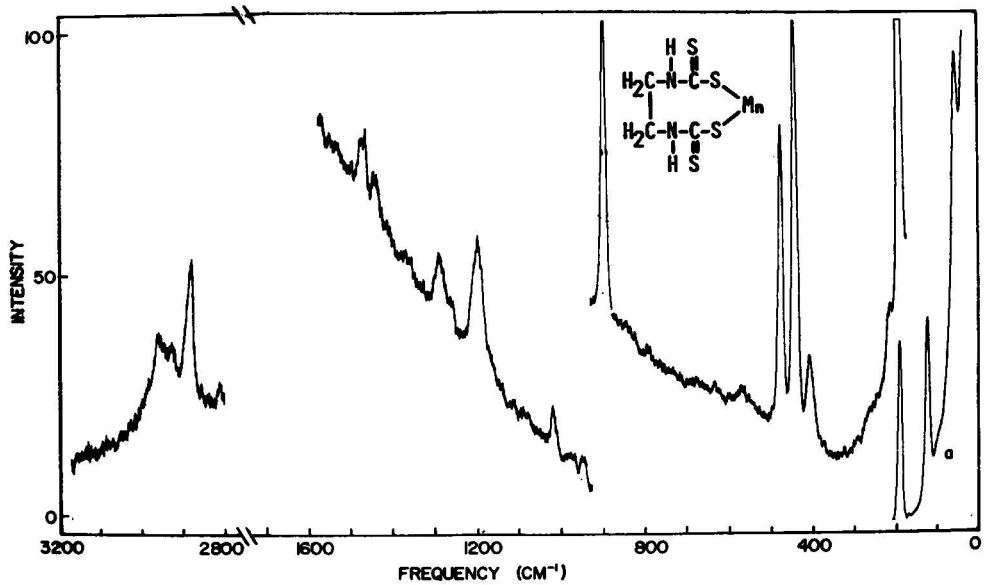


FIG. 9—Raman spectrum of zineb. a, sensitivity ~0.21×.

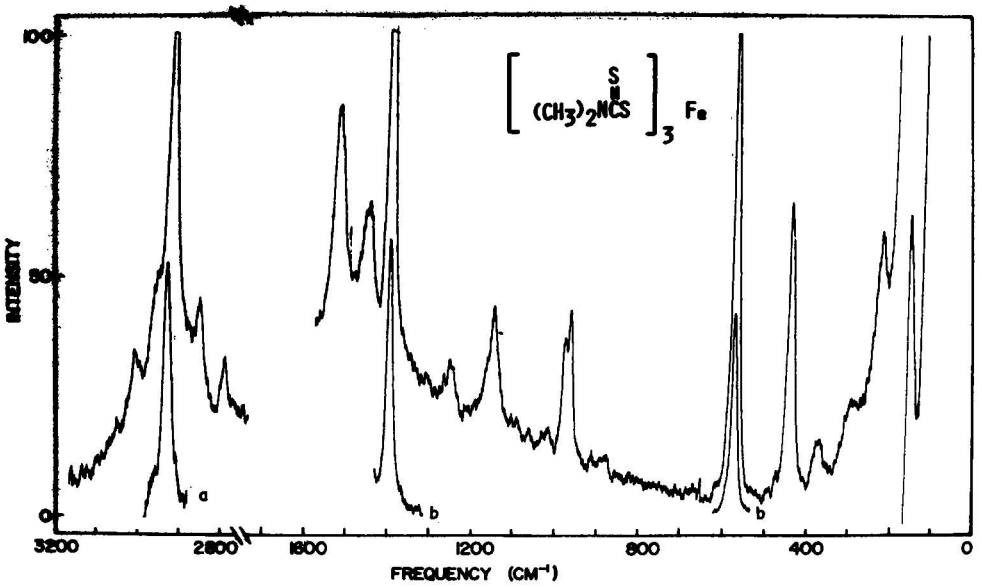


FIG. 10—Raman spectrum of ferbam. a, sensitivity ~0.67×; b, sensitivity ~0.53×.

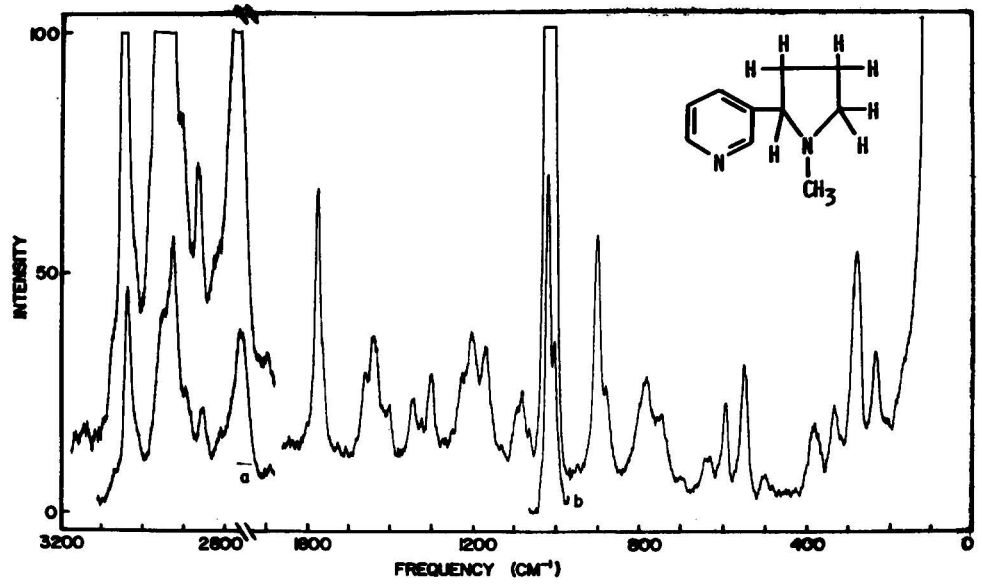


FIG. 11—Raman spectrum of nicotine. a, sensitivity ~0.42×; b, sensitivity ~0.21×.

even in the face of a sizable mass difference between manganese and zinc, is strong evidence that the bonding between the metal and the organic portion of these molecules is ionic in nature and not covalent.

The Raman spectrum of nicotine was earlier reported by Brustier and Blanc (6), using mercury arc excitation. Not surprisingly, we were able to record many more lines than they did. Aside from a cluttered and complicated C—H stretching region, the most important features of the spectrum are the strong bands at 1584, 1036, and 928 cm^{-1}. The first 2 of these are associated with the pyridine ring (5) and the third is probably due to the pyrrolidine ring (6).

In this series of 3 papers, we have tried to point out certain features of the Raman spectra of pesticides which might make this type of spectroscopy useful to the analyst working with pesticides. Clearly, we have barely begun the problem as we have dealt neither with residue studies nor with actual field samples. However, we still feel Raman spectroscopy offers possibilities in this area and that studies of this type should be pursued.

Financial support from the National Science Foundation through Undergraduate Research Participation Grants GY-11159 and EPP 75-04306 and from Research Corporation for the purchase of the Raman spectrometer was received.

Acknowledgment

We wish to thank Sarah R. Huff for her help during this project.

REFERENCES

(1) Nicholas, M. L., Powell, D. L., Williams, T. R., & Bromund, R. H. (1976) *JAOAC* **59**, 197–208

(2) Nicholas, M. L., Powell, D. L., Williams, T. R., & Huff, S. R. (1976) *JAOAC* **59**, 1071–1080

(3) Frear, D. E. H. (1969) *Pesticide Index*, 4th Ed., College Science Publishers, State College, PA

(4) Kohlrausch, K. W. F., & Pongratz, A. (1934) *Z. Phys. Chem.* **B27**, 176–195

(5) Rao, C. N. R., & Venkatarazhavan, R. (1962) *Spectrochim. Acta*, **18**, 541–547

(6) Brustier, V., & Blanc, P. (1943) *Bull. Soc. Chim. Fr.* **10**, 58–59

A Simple Spectrophotometric Method for the Determination of Carbofuran Residues

JOIS R. RANGASWAMY, YADATHORA N. VIJAYASHANKAR, and SHIMOGA R. PRAKASH
Central Food Technological Research Institute, Mysore 570013, India

A method has been developed for carbofuran residues, based on coupling carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methyl carbamate) with diazotized aniline to form a yellow compound with an absorption maximum at 460 nm. The relationship between absorbance and concentration is linear for 1–10 μg carbofuran/5 ml. The method is sensitive and can be applied to the determination of levels as low as 0.025 ppm carbofuran in a 40 g crop or soil sample.

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methyl carbamate) is a broad spectrum pesticide which has demonstrated excellent biological activity against pests on a wide variety of crops. Soil-incorporated applications have provided effective control of root pests. It is effective as both a systemic and a contact toxicant and is less persistent than chlorinated pesticides. It is non-corrosive and compatible with other pesticides. Butler and McDonough (1) have reported an electron capture gas-liquid chromatographic (GLC) method for determining carbofuran residues in potatoes, sugar beets, apples, and orange grass. The phenol obtained by the hydrolysis of carbofuran was heated with pyridine and trichloroacetyl chloride. The resulting trichloroacetyl derivative, after cleanup on a Florisil column, was used for GLC determination. Bowman and Beroza (2) used the dimethyl chlorothiophosphate derivative of carbofuran in a GLC method for determining carbofuran residues in corn silage and milk. A cholinesterase inhibition method was used by Dale (3), while Cook *et al.* (4) and Williams and Brolin (5) described a GLC technique with a nitrogen-specific microcoulometric detector. As cited by Kapoor and Kalra (6), Gupta and Dewan have developed a thin layer chromatographic method for determining carbofuran residues; *p*-nitrobenzenediazonium fluoroborate spray reagent reacts with the phenol of the hydrolyzed carbamate to give a reddish color. The simple method described below is based on the electrophilic reaction of diazotized aniline with carbofuran.

METHOD

Reagents

All solvents are analytical reagent grade.

(a) *Aniline.*—Distilled (British Drug Houses, London, England). Dissolve 50 mg aniline in 1 L 0.1*N* HCl.

(b) *Carbofuran.*—Analytical reagent grade (Niagara Chemicals Division, FMC Corp., Middleport, NY). Prepare standard solutions containing 1–10 μg carbofuran/ml methanol.

(c) *Carbofuran formulation.*—50% dust (Indofil Chemicals Ltd., Bombay, India).

(d) *Silica gel.*—Column grade (National Chemical Laboratory, Poona, India). Prepare slurry of 8 g silica gel in $CHCl_3$ and pack into 1 cm id glass column to ca 20 cm height.

Preparation of Standard Curve

Pipet 1 ml aniline solution into separate clean test tubes. Diazotize by adding 0.5 ml 5% $NaNO_2$ to each test tube. Add 1 ml aliquots of each carbofuran standard solution to separate test tubes. Shake well and add 1 ml 0.5*N* KOH to each. Shake tubes well and let stand 30 min at room temperature. Dilute resulting yellow solutions to 5 ml with methanol and read at 460 nm against blank prepared similarly. Plot of concentration vs. absorbance will yield straight line.

Determination

Crops.—Extract 40 g well minced and fortified samples (both green and red tomato, okra (lady's finger), cowpea, and their plants) twice with 30 ml $CHCl_3$ or methylene chloride. Filter organic layer into evaporating dish and evaporate to dryness at room temperature (methylene chloride) or on water bath ($CHCl_3$). Dissolve residue in known volume of acetonitrile and extract solution with 2–3 portions of 20 ml hexane. Prepare crop control similarly. Use 1 ml aliquot of acetonitrile solution for color development as described under *Preparation of Standard Curve.* If colored solution is turbid or has an emulsion, centrifuge 20 min at 2000 rpm before taking readings. Re-extract green $CHCl_3$ extract of okra with two 20 ml portions of water to dissolve and remove mucilaginous material.

Soil.—Mix 40 g sample of dry soil with 12–15

ml water (ca 30% moisture level). Extract pasty mass twice with 20 ml $CHCl_3$, and filter into evaporating dish. Evaporate $CHCl_3$ to dryness, and dissolve residue in known volume of acetonitrile. Prepare control sample similarly. Use 1 ml aliquot (without hexane extraction) for color development.

Residue Analysis

Spray pot plants at recommended dosages with water dispersion of 50% dust formulation of carbofuran, and use for analysis. Separately analyze plant and fruit portions of tomato and cowpea for carbofuran residues. Extract representative samples of well chopped crop with methylene chloride and evaporate to dryness at room temperature as above. Since 50% dust formulation is red due to presence of dye, residue obtained after evaporation of methylene chloride is red. Add $CHCl_3$ solution of residue to silica gel column and elute carbofuran with 150 ml $CHCl_3$. Dye remains on top of silica gel column. Concentrate $CHCl_3$ eluate to dryness, and dissolve colorless residue in known volume of methanol. Use aliquot of solution for color development as described under *Preparation of Standard Curve*.

Results and Discussion

To check the recovery of carbofuran by the method described, samples were fortified with known amounts of carbofuran and were analyzed. The recoveries varied from 90 to 100% (Table 1). The method can be applied to the analysis of soil treated with carbofuran. Since no interfering materials are encountered either from the fruit or the plant, the method can be applied to determining carbofuran translocated into plant tissue from treated soil. The method is sensitive and can be used to determine levels as low as 0.025 ppm in a 40 g sample (permissible limit is 2 ppm). The method has also been used to determine carbofuran residues on pot plants sprayed with a water dispersion of a 50% dust formulation (Table 2). Compared to other methods reported, the present method does not rely on the presence of free hydroxyl groups, so hydrolysis of methyl carbamate prior to estimation is not necessary. It also does not involve elaborate methods of cleanup and hydrolysis to purify the crop extracts. Hence higher recoveries of the residues are obtained because losses due to different steps of cleanup procedures are avoided. As in the GLC method, neither derivatization nor elaborate purification is necessary.

Table 1. Recovery of carbofuran from fortified samples

Added, μg	Found, μg[a]	Rec., %
	Green Tomato	
50.0	48.33±0.11	97
25.0	24.17±0.02	97
10.0	10.00±0.01	100
	Red Tomato	
50.0	49.16±0.11	98
30.0	29.66±0.18	99
20.0	19.00±0.10	95
	Tomato (Plant)	
50.0	49.05±0.20	98
30.0	29.02±0.50	99
20.0	18.92±0.10	95
	Okra	
50.0	48.00±0.00	96
30.0	30.00±0.00	100
20.0	18.00±0.00	90
	Okra (Plant)	
30.0	29.12±0.70	97
20.0	18.89±0.90	94
	Cowpea	
100.0	98.12±0.90	98
50.0	48.88±0.40	98
25.0	23.89±0.70	96
	Soil	
75.0	72.00±0.02	96
40.0	36.45±0.02	91
20.0	18.00±0.00	90

[a] Average of 6 determinations ± standard deviation.

Table 2. Residue analysis of pot plants sprayed with dispersion of carbofuran formulation

Active ingredient, mg/plant	Residue found, ppm[a]	
	Plant	Fruit
	Tomato	
82.0	188.0±0.08	16.15±0.06
41.0	98.6±0.12	7.66±0.02
	Cowpea	
3.8	62.5±0.00	8.15±0.05
1.9	32.5±0.01	4.58±0.09

[a] Average of 6 determinations ± standard deviation.

The linear relationship between the absorbance at 460 nm and the concentration of carbofuran is valid up to 10 μg/5 ml reaction mixture. Beyond this level the relationship is not linear and inconsistent recoveries are obtained. If a higher carbofuran residue is detected during analysis, a larger volume of acetonitrile must be used to dissolve the residue in the evaporating dish, so that the concentration of the residue/ml is within the limit of Beer's law. Maximum color development takes place in 30 min and the color is stable for about 2 hr. A methanolic solution of carbofuran should be used, because erratic values are obtained when ethanol is used as solvent. Methanol should also be used for diluting the colored solution to 5 ml, as turbidity results when water is used due to insolubility of the color compound. Alkali stronger than 0.5*N* renders the blank intense yellow.

Acknowledgment

The authors thank B. L. Amla for kind encouragement and S. K. Majumder for critical appraisal of the problem.

REFERENCES

(1) Butler, L. I., & McDonough, L. M. (1968) *J. Agric. Food Chem.* **16**, 403–407
(2) Bowman, M. C., & Beroza, M. J. (1967) *JAOAC* **50**, 926–933
(3) Dale, D. (1975) *Pesticides* **9**, 25–28
(4) Cook, R. F., Stanovick, R. P., & Cassil, C. C. (1969) *J. Agric. Food Chem.* **17**, 277–282
(5) Williams, I. H., & Brolin, M. J. (1973) *J. Agric. Food Chem.* **21**, 399–401
(6) Kapoor, S. K., & Kalra, R. L. (1975) *J. Food Sci. Technol.* **12**, 227–230

Received May 18, 1976.

Comparison of Dieldrin, Lindane, and DDT Extractions from Serum, and Gas-Liquid Chromatography Using Glass Capillary Columns

JOSEF J. FRANKEN and BERT J. M. LUYTEN
Laboratory of Instrumental Analysis, Eindhoven University of Technology, Eindhoven, The Netherlands 4500

Rats were given an oral dose of ^{14}C-labeled chlorinated pesticides to obtain serum containing *p,p'*-DDT, dieldrin, or lindane. Simple hexane and formic acid-hexane extraction methods, involving pretreatment of the serum with formic acid, were compared by radiometric and by paper chromatographic and gas chromatographic analysis. *In vivo* binding of chlorinated pesticides to constituents of the serum does not necessarily prohibit their isolation by simple hexane extractions, provided that the extraction is very vigorous and at least 5 min long. Stable emulsions were broken by cooling in liquid nitrogen or Dry Ice-acetone. The hexane extraction method described yields quantitative recovery of the pesticides studied, whereas the formic acid-hexane method is quantitative for *p,p'*-DDT, 93% for dieldrin, and 89% for lindane. Gas chromatographic comparison of both methods, using human serum, shows that the hexane method extracts 16% more β-BHC, 7% more dieldrin and HCB, and 4% more *p,p'*-DDE from serum than does the formic acid-hexane method. The difference for *p,p'*-DDT is not significant. Gas chromatography with glass capillary columns and an all-glass solids injection system yielded detection limits as low as 15 fg. Data show that the use of an internal standard considerably improves the precision of quantitation.

A functional dependence exists between the concentrations of chlorinated pesticides in various tissues and that in blood or serum. At a state of equilibrium between intake and elimination, a steady state of storage is reached with a constant total body burden in dynamic equilibrium with a corresponding constant blood level and constant levels in other tissues of the body. Thus, pesticide blood levels are a suitable measure of the pesticide body burden and reflect the level of chronic exposure to pesticides. Determinations of the pesticide blood level are an easily used and extremely valuable technique for the study of the effects of pesticides in man (1).

Although analytical methods for pesticides in serum have gained a lot of attention, there is no clear agreement on the efficiency of the numerous different extraction methods. Efficiency is normally determined *in vitro* under the assumption that *in vitro* added pesticides behave in the same manner as do pesticides incorporated via metabolic pathways. However, the only way to arrive at unequivocal results is to use radiolabeled material metabolically incorporated in the serum at concentration levels comparable to those in the samples. In this paper we describe a series of experiments devised to unambiguously determine the efficiency of some isolation methods for *p,p'*-DDT, dieldrin, and lindane.

The hexane extraction methods described by Dale *et al.* (2) and Radomski and Rey (3) yield clean and colorless samples which can be analyzed without cleanup. An important disadvantage of these methods is the frequently observed formation of a stable emulsion. This problem was overcome by a modification proposed by Franken (4), involving treating the sample in liquid nitrogen or in Dry Ice-acetone. According to Dale *et al.* (5), the formation of stable emulsions is also avoided by adding formic acid to the serum before extraction with hexane. Under certain precautions (c.f., *Experimental*) this method yields extracts which can be analyzed without extensive cleanup. We chose to consider both these methods in more detail.

In the original paper on hexane-extractable chlorinated pesticides, Dale *et al.* (2) found that recovery was not complete. Bonderman *et al.* (6) proposed procedural modifications to optimize quantitative results. Chiba and Morley (7) indicated losses due to volatilization during evaporation were a major source of error. To improve extraction efficiencies, polar solvent mixtures are frequently used, but Radomski and

Received January 30, 1976.

Rey (8), in a series of comparative experiments, arrived at the conviction ". . . that the observation that other more polar solvents and solvent mixtures extract more pesticides than hexane from blood derives from a failure to shake the blood and hexane sufficiently during the extraction." Dale *et al.* (5) compared the formic acid-hexane method with the hexane extraction method. Efficiencies for total DDT-derived materials obtained by the formic acid method were quantitative, in contrast to those for the hexane method. However, in a recent interlaboratory collaborative study (9), the results obtained with the formic acid method by independent participants proved to be highly controversial and were far from quantitative. Apparently, in the literature there is no unanimous opinion, either on the efficiency of the hexane extraction or on the efficiency and feasibility of the formic acid extraction. In this paper we investigate and compare extraction methods, based on the hexane and the formic acid extractions, but modified so as to take into account the results and recommendations of Bonderman *et al.* (6), Chiba and Morley (7), and Franken (4). Furthermore we discuss some aspects of the gas-liquid chromatographic (GLC) quantitation at the (sub-) picogram level, based on the use of high-resolution glass capillary columns and an all-glass solids injection system.

Experimental

Apparatus and Reagents

(a) *Gas chromatograph.*—A Pye Unicam Series 104 Model 84 was used, equipped with 10 mCi ^{63}Ni electron capture detector. The injection port was removed and replaced by an all-glass solids injection system (10), which was modified as described below. Separations were carried out on glass capillary columns of relatively wide bore, prepared according to Franken and Vader (11) and Franken and Rutten (12). Typically, columns were 25 m × 0.5 mm id, 1.2 mm od, coated according to Bouche and Verzele (13) with 0.6 μm thick SE-30 or OV-1 stationary phase film. Coating efficiencies as defined by Ettre (14) ranged from 80 to 95% for *p,p'*-DDT at 220°C column temperature. A length of Dilver P metal capillary was melted to the exit end of the capillary column, according to Vidal-Madjar *et al.* (15). This metal capillary leads the effluent to a point just below the ^{63}Ni foil, thus avoiding the influence of dead space and adsorption in the interconnecting tubing. All connections were made with shrinkable PTFE tubing. Operating conditions normally were: inlet pressure 0.35 kg/sq cm, argon-methane (95+5) carrier gas, column temperature 220°C, detector temperature 300°C, argon-methane (95+5) detector purge gas flow 25 ml/min as measured at room temperature.

(b) *Liquid scintillation spectrometer.*—Packard Tricarb Model 2311; Instagel® scintillator (Packard Instrument Co.).

(c) *^{14}C-Labeled pesticides.*—Lindane, specific activity 155 μCi/mg, radiochemical purity 99%, dieldrin, specific activity 91 μCi/mg, radiochemical purity 99%, and *p,p'*-DDT, specific activity 144 μCi/mg, radiochemical purity >97% were obtained as benzene solutions from the Radiochemical Centre, Amersham, England. Fifty μl sunflower oil was added to each of the pesticide solutions, the benzene was removed under a gentle stream of nitrogen at 60°C, and the residues were vigorously shaken with 1.5 ml milk each until stable emulsions were obtained. (Purchase, use, and disposal of radiolabeled material is subject to statutory restrictions and safety regulations.)

(d) *Centrifuge tubes.*—Pyrex, or equivalent low thermal expansion glass.

(e) *n-Hexane.*—Uvasol® (E. Merck, Darmstadt, West Germany).

(f) *Formic acid.*—97% (E. Merck); shake twice with excess *n*-hexane shortly before use.

(g) *Aqueous potassium carbonate.*—Shake 5% aqueous solution twice with equal volumes of *n*-hexane.

Animal Experiments

Nine adult male Wistar rats, weighing 380–450 g, were dosed by stomach tube with 0.5 ml milk each, containing one of the radiolabeled pesticides. Dosage levels were: 0.4 mg DDT/kg body weight; 0.3 mg dieldrin/kg; 0.05 mg lindane/kg. Three rats were used for each of the 3 pesticides under study. After 4 hr, the rats were sacrificed by exsanguination. Ether was used for anesthetics. Serum was obtained in the usual way. Sera containing the same pesticide were combined and stored at −20°C. This gave ca 10 ml serum for each pesticide.

Extraction

Four extraction procedures were carried out for each pesticide, using an extraction setup analogous to that of Sundaram and Connell (16).

Extraction A.—Vigorously shake 1 ml serum in centrifuge tube with 0.5 ml *n*-hexane for 5 min, using Vortex mixer. Centrifuge jelly-like emulsion 2 min at 2000 rpm and slowly immerse tube into liquid nitrogen or Dry Ice-acetone until contents of tube solidify. (*Caution:* Avoid contacting skin

with freezing mixture or cooled tube.) Warm tube to room temperature in lukewarm water bath and centrifuge again 1 min. Carefully remove upper layer with disposable pipet and store in second tube. Again pipet 0.5 ml *n*-hexane into tube containing serum and repeat extraction and cooling as described. Combine extracts.

Extraction B.—Extract 1 ml serum once with 1 ml *n*-hexane for 5 min, centrifuge, cool, and centrifuge again as described under A.

Extraction C.—Pipet 1 ml serum plus 1 ml 97% formic acid into centrifuge tube and shake 1 min on Vortex mixer. Add 0.5 ml *n*-hexane and shake vigorously 5 min on Vortex mixer. Let phases separate, and centrifuge 1 min. Carefully remove upper layer with disposable pipet and place in second tube. Again pipet 0.5 ml *n*-hexane into tube containing serum and repeat extraction as described. Combine extracts, and shake combined extracts once with 1 ml 5% potassium carbonate 1 min.

Extraction D.—Mix 1 ml serum and 1 ml formic acid as described under C. Extract mixture once with 1 ml *n*-hexane 5 min. Let layers separate, and centrifuge 1 min. Remove upper layer with disposable pipet and place in second tube. Shake extract once with 1 ml 5% potassium carbonate 1 min.

Paper Chromatography

Both the aqueous and organic layers may contain radiolabeled metabolites. To calculate extraction efficiencies accurately, the percentage radioactivity present in non-metabolized parent compounds was determined. Metabolites were separated from parent compounds by paper chromatography, essentially according to Mills (17), using methanol-water as mobile solvent. Organic samples were applied to paper as separate dots; aqueous samples were applied as series of overlapping dots over full width of paper to obtain distribution of sample as evenly as possible. Separated compounds were detected and quantitated radiometrically. The paper chromatogram was cut breadthwise to 1 cm wide strips, starting 0.5 cm below the origin line. Each strip was placed in counting vial and shaken with Instagel until translucent. The radioactivity content per strip was determined by liquid scintillation counting.

Gas Chromatography

To validate the results obtained with rat serum, human serum was carried through extraction procedures B and D and analyzed by gas chromatography. The 2 methods were compared and the differences were related to the differences found by the radiometric measurements on rat serum. From replicate analyses of extracts, the precision of the proposed GLC method was determined. The precision of the entire method, involving extraction procedure B, was estimated from replicate extractions.

Results and Discussion

In the animal experiments ether was used for anesthetics because this is not bound to serum albumins and does not influence the equilibrium between free pesticides and pesticides attached to serum albumins. We consider that the final results are not influenced by the presence of traces of ether in the serum. The results of the paper chromatographic analysis of the original sera before extraction are given in Fig. 1. Although the rats were bled only 4 hr after dosing, it follows that an appreciable part of the serum radioactivity content is present in radiolabeled metabolites, viz. 21% in DDT metabolites, 6% in dieldrin metabolites, and 48% in lindane metabolites. An explanation might be found in different rates of disappearance from the blood of pesticides and metabolites. The highly lipophilic pesticides are much more effectively immobilized in adipose tissues than are the metabolites, while the rate of excretion of the metabolites is relatively low. Hence, during the first few hours after dosing, the blood is relatively rich in metabolites, making a correction for metabolism in *in vivo* experiments inevitable.

After correction for metabolism, the serum radioactivity content gave the chemical concentration of the radiolabeled pesticides; DDT was present at 82 ng/ml, dieldrin at 28 ng/ml, and lindane at 5 ng/ml.

Extraction results are given in Table 1. Radioactivity extraction efficiency (R') was calculated from the total radioactivity content of the layers, hence, it relates to total radiolabeled material. The pesticide balance (b) and the pesticide extraction efficiency (R) were calculated after multiplying the radioactivity content of each layer by the fraction of non-metabolized pesticide (F) therein (not given in the table).

In some cases the volume of sample available for paper chromatography was too small to carry out a precise determination of the value of F. A minimum or maximum value then was estimated and this estimate was used in the calculations. In the table this is indicated by signs $\geqslant$ or $\leqslant$.

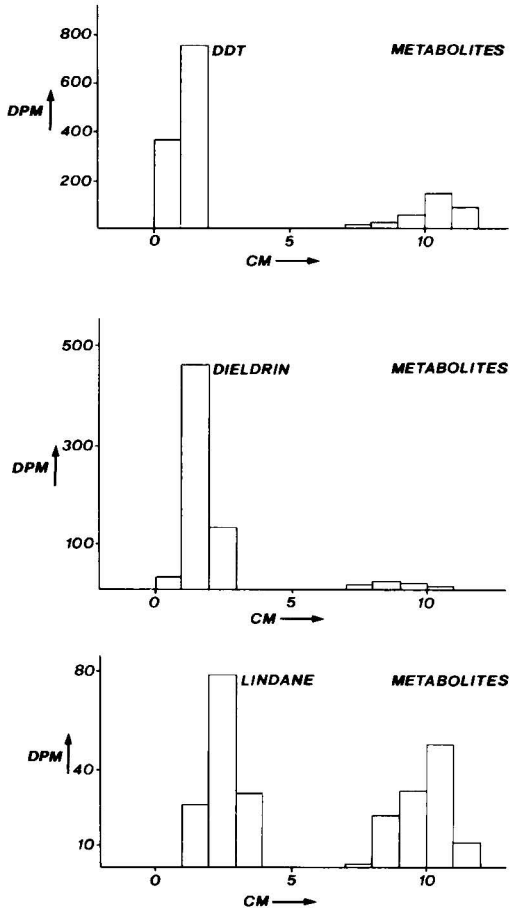


FIG. 1—Bar graphs showing results of paper chromatographic analysis of rat serum containing ^{14}C-labeled pesticides, before extraction. Bars represent net radioactivity content per 1 cm wide strip of paper chromatogram.

Deviations of b from the ideal value of 100% are a measure of the experimental error involved. Both volumetric errors, counting errors, and errors in the value of F are accounted for in the value of b. Since b is very close to 100% in most cases, the uncertainty in the values of R is estimated to be 3% or less. The widely different values of R and R' once again stress the importance of a correction for metabolism in *in vivo* experiments.

Comparing the calculated extraction efficiencies R, it is apparent that the differences between the hexane extraction and the formic acid-hexane extraction are only marginal. The increased efficiency for p,p'-DDT, using the formic acid method, is not significant; neither is the decreased efficiency found for dieldrin. Only in the case of lindane are the differences significant; the hexane method yields near to quantitative results, whereas formic acid extracts only 89% in extraction D and 82% in the first step of extraction C.

Results of a GLC comparison of the hexane (extraction B) and formic acid (extraction D) methods for determining pesticides in human serum are given in Table 2. The difference in the results as obtained by the 2 methods for p,p'-DDT is not significant. For p,p'-DDE the difference is of doubtful significance ($P = 0.9$). For dieldrin, β-BHC, and hexachlorobenzene (HCB), significantly higher results were obtained by the hexane method than by the formic acid-hexane method. For dieldrin and p,p'-DDT, the differences as measured by GLC compare very well with the differences expected, as calculated from the efficiencies given in Table 1. Apparently, results obtained using rat serum may be valid for human serum too.

The experiments described above were performed under stringent precautions to reduce errors. Relative standard deviations of the final results do not exceed 2%. Relative standard deviations of the mean of duplicate analyses, as observed in day-to-day practice, are present in Table 3, as are the concentration levels at which these values were measured. Serum of a non-occupationally exposed individual was carried through extraction procedure B. The relative standard deviation due to GLC was calculated from repetitive injections of the extract. Results were quantitated by peak height measurements, with and without the use of 4 ng aldrin/ml as internal standard. If the presence of aldrin at the ppb level could not be ruled out, 2,2′-dibromobiphenyl was used as internal standard. The retention of 2,2′-dibromobiphenyl on SE-30 stationary phase, relative to aldrin, is 0.51 at 220°C. The internal standard method appears to reduce quantitation errors considerably, yet it was not used to advantage in most methods reported earlier. The relative standard deviation due to GLC plus extraction was calculated from repetitive extractions. Prior to extraction, 4 ng internal standard/ml hexane was added. The injected volume was 1 μl in all cases.

Table 1. Comparison of extraction efficiencies obtained by hexane and formic acid-hexane extraction procedures[a]

Extraction	*p,p'*-DDT			Dieldrin			Lindane		
	b	*R'*	*R*	*b*	*R'*	*R*	*b*	*R'*	*R*
Hexane Extraction									
A, first extn	≤98	72	≥94	≤103	92	≥97	100	50	≥95
first + second extn	97	74	99	≤103	95	100	100	50	100
B	97	72	97	104	94	98	98	51	100
Formic Acid-Hexane Extraction									
C, first extn	100[b]	83	97	≥101	91	≤94	103	48	82
first + second extn	101[b]	87	100	102	94	≥96	—	57	—
D	101[b]	86	99	104	91	93	107	50	89

[a] *b* = radiolabeled pesticide balance, %. *R'* = extraction efficiency of total radioactive material, %; *R* = extraction efficiency of radiolabeled pesticide, %.
[b] Total radioactive materials balance.

Table 2. Comparison of hexane and formic acid-hexane extractions for determination of pesticides in serum of general population

Compound	Concn, ng/ml	Diff.,[a] %
β-BHC	3	−16
HCB	9	− 7
Dieldrin	1	− 7
p,p'-DDE	24	− 4
p,p'-DDT	5	+ 2

[a] Calculated from ((FH-HE)/HE) × 100%, where FH refers to formic acid-hexane extraction procedure D and HE refers to hexane extraction procedure B; *n* = 3.

Table 3. Precision of GLC quantitation and hexane extraction procedure B for pesticides in serum of a non-occupationally exposed individual

Compound	Mean x̄4, ng/ml	Rel. std dev. of mean of dupl. anal., %, *n* = 4		
		GLC, no int. std	GLC, int. std	GLC + extn
β-BHC	1.11	6.4	1.0	5.0
HCB	5.93	2.4	4.1	5.7
γ-BHC (lindane)	0.84	8.7	1.5	5.1
Dieldrin	0.34	9.8	1.5	3.7
p,p'-DDE	8.82	5.0	2.2	3.0
p,p'-DDD[a]	1.08	7.9	1.0	1.3
p,p'-DDT[a]	1.45	7.3	0.3	4.2

[a] Peak heights rounded to next 0.5 mm, thus seemingly reducing relative standard deviation of small peaks.

A typical chromatogram is reproduced in Fig. 2. The signal of α-BHC corresponds to 30 fg (femtogram), which is 2 times the detection limit of 15 fg for the BHC isomers and HCB. For DDT-derived compounds (e.g., *o,p'*-DDT, peak No. 7) and for dieldrin, the detection limit is about 0.1 pg.

Conclusions

Our results prove that binding of pesticides to constituents of the serum does not prohibit their extraction by hexane. The hexane method is fully satisfactory, provided that experimental parameters of storage and extraction are optimized. To obtain consistently high efficiencies, it is especially important that the extraction is vigorous and at least 5 min long. Stable emulsions can be broken by cooling in liquid nitrogen or Dry Ice-acetone. A single extraction of 2 volumes of serum with as little as one volume or more of hexane will yield a near to quantitative recovery. By using these small hexane volumes, evaporation of extracts, known to be a major source of error, can be omitted. Aliquots of the hexane layer are analyzed without phase separation. The addition of an internal standard to the solvent prior to extraction enhances the precision of quantitation. The method as a whole is extremely simple and rapid, requiring a minimum of glassware and solvents and thus reducing the opportunities for losses and contamination to a minimum.

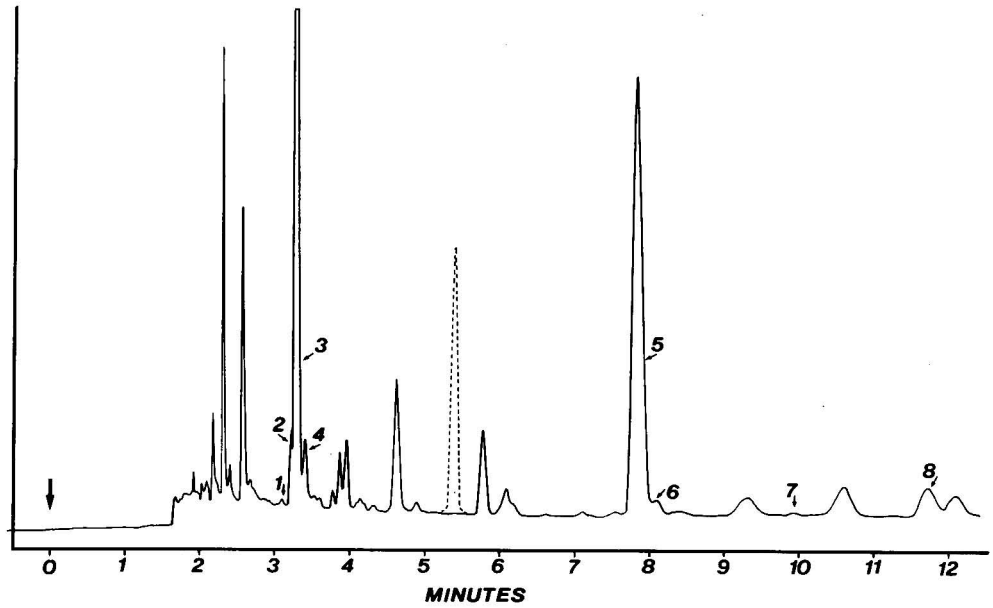


FIG. 2—Gas chromatogram of hexane extract (B) of serum of a non-occupationally exposed individual.

Column: 31 m × 0.6 mm id Pyrex capillary, coated with 0.6 μm SE-30, 0.6 μl injection volume. For conditions see text. 1, 0.05 ppb α-BHC (2 × detection limit); 2, 0.7 ppb β-BHC; 3, 5.2 ppb HCB; 4, 0.5 ppb γ-BHC; 5, 15 ppb *p,p'*-DDE; 6, 0.5 ppb dieldrin; 7, 0.2 ppb *o,p'*-DDT (detection limit); 8, 1.4 ppb *p,p'*-DDT. No internal standard injected. Dashed peak, 4 ppb aldrin internal standard, superimposed.

The combination of the high resolving power and low catalytic activity of glass capillary columns with the extreme sensitivity of the ^{63}Ni electron capture detector proves to be a powerful tool for the analysis of (sub)-ppb levels of chlorinated pesticides. Detection limits as low as 15 fg can be obtained.

Acknowledgments

The authors record their gratitude to H. M. van Praag, Psychiatric University Clinic, Groningen, who offered the opportunity to perform the animal experiments and to F. Postema for technical assistance.

REFERENCES

(1) Radomski, J. L., Astolfi, E., Deichman, W. B., & Rey, A. (1971) *Toxicol. Appl. Pharmacol.* **20**, 186–193

(2) Dale, W. E., Curley, A., & Cueto, C., Jr. (1966) *Life Sci.* **5**, 47–54

(3) Radomski, J. L., & Rey, A. (1970) *J. Chromatogr. Sci.* **8**, 108–114

(4) Franken, J. J. (1973) in *Symposium VII Chromatographie Electroforese,* Presses Academiques Europeenes, Brussels, Belgium, pp. 441–452

(5) Dale, W. E., Miles, J. W., & Gaines, T. B. (1970) *JAOAC* **53**, 1287–1292

(6) Bonderman, D. P., Choi, U. Y., Hetzler, H. L., & Long, K. R. (1969) *JAOAC* **52**, 1063–1066

(7) Chiba, M., & Morley, H. V. (1968) *JAOAC* **51**, 55–62

(8) Radomski, J. L., & Rey, A. A. (1970) *Pestic. Symp. Collect. Pap. Inter-Amer. Conf. Toxicol. Occup. Med., 6th, 7th 1968–1970,* pp. 67–73

(9) Stretz, P. E., & Stahr, H. M. (1973) *JAOAC* **56**, 1173–1177

(10) van den Berg, P. M. J., & Cox, T. P. H. (1972) *Chromatographia* **5**, 301–305

(11) Franken, J. J., & Vader, H. L. (1973) *Chromatographia* **6**, 22–27

(12) Franken, J. J., & Rutten, G. A. F. M. (1973) in *Chromatography 1972,* S. G. Perry (Ed.), Applied Science Publishers Ltd., London, England, pp. 75–88

(13) Bouche, J., & Verzele, M. (1968) *J. Gas Chromatogr.* **6**, 501–505
(14) Ettre, L. S. (1959) *Open Tubular Columns in Gas Chromatography,* Plenum Press, New York, NY, p. 15
(15) Vidal-Madjar, C., Ganansia, J., & Guiochon, G. (1971) in *Gas Chromatography 1970,* R. Stock (Ed.), Institute of Petroleum, London, England, pp. 20–34
(16) Sundaram, K., & Connell, K. G. (1975) *Anal. Chem.* **47**, 779–780
(17) Mills, P. A. (1959) *JAOAC* **42**, 734–740

CHANGES IN ADDRESS

Associate and General Referees—Has your address changed since the list of Officers and Standing Committees of the AOAC was published in the March issue of *JAOAC*? This list is used as the basis of a mailing list—to notify people of items of interest and also to remind people of the deadline for reports and papers to be presented at the AOAC meeting. It is essential that this list be kept up to date. If your address has changed, please notify the AOAC Business Office.

DRUGS IN FEEDS

Collaborative Study of a Modified Extraction Procedure for Lasalocid Sodium in Feeds by Microbiological Assay

JACOB M. SCHEINER
Product Development Department, Hoffmann-La Roche Inc., Nutley, NJ 07110

A collaborative study was conducted on a modified extraction technique in the microbiological determination of lasalocid sodium in finished poultry feeds. The feed is warmed briefly with pH 4.7 buffer and the drug is extracted with ethyl acetate. The extract is washed with HCl and NaOH and evaporated to dryness, and the residue is dissolved in hexane, partitioned into methanol-water (3+1), and diluted to assay range at a final alcohol concentration of 25% (v/v). Seven laboratories participated in the study. Average recoveries of lasalocid sodium added at 0.01035, 0.00863, and 0.0069% ranged from 93 to 99% for mash feeds and from 86 to 95% for pellets. The coefficients of variation averaged 11.2% for mashes and 9.8% for pellets. The modified extraction technique has been adopted as official first action.

The 1974 AOAC collaborative study (1) of the microbiological assay of feeds for the coccidiostat lasalocid sodium (Avatec™, antibiotic X-537A) included recovery tests in which lasalocid sodium was added to unmedicated mash and pellets. Satisfactory results were obtained by correcting the assays of the medicated feeds by the corresponding recovery factor. In subsequent studies of various modifications of the extraction step, the procedure of Osadca and Araujo (2) yielded excellent recoveries, which eliminated the need for recovery tests. In this procedure, the feed sample is warmed with pH 4.7 buffer prior to extraction with ethyl acetate. The extract is washed with hydrochloric acid and sodium hydroxide before the ethyl acetate is evaporated. This procedure was subjected to collaborative testing.

Collaborative Study

A typical commercial broiler starter-grower mash was used to prepare feed mixes containing 69 (Mix 1), 86.3 (Mix 2), and 103.5 (Mix 3) ppm lasalocid sodium added as a 15% premix. The mash feeds were thoroughly mixed and finely ground in a Model D Fitzpatrick mill, using No. 2 perforated plates to enhance uniformity of drug distribution. Samples of pelleted feeds were similarly prepared.

Each collaborator was supplied with the following: 6 mash and 6 pellet test samples coded Mix 1—A and B, Mix 2—A and B, Mix 3—A and B, where A and B represented duplicate samples of the same feed mixture; a practice mash feed sample containing about 80 ppm lasalocid sodium; lasalocid sodium reference standard; a copy of the assay method; a stock spore suspension of *Bacillus subtilis* ATCC 6633; and a set of report forms. The participants were instructed to analyze the practice mash in duplicate and, if low values were obtained for this sample, to purify the ethyl acetate by passage over a silica gel column, followed by distillation. They were then requested to perform a single assay on each test sample, using mash samples as received and pellets after grinding. Samples labeled A (3 mashes and 3 pellets) were assayed on one day and the parallel samples labeled B were assayed on a second day. Time permitting, the collaborators were requested to evaporate some of the extracts under air or under vacuum to compare to results obtained with nitrogen.

METHOD

The microbiological cylinder plate assay for the determination of lasalocid sodium in premixes and feeds, **42.A01–42.A07**, was modified as follows:

(a) Change line 1 of **42.A01** to read: "*See* **42.194(a)–(c)**, **42.A10(a)**, and following:".

(b) In **42.A02(b)**, line 2, change "with aq. MeOH, (c)," to "using anhyd. MeOH and H_2O,".

(c) In **42.A02(b)**, line 3, change "3.0 μg/ml" to "4.0 μg/ml".

(d) Change **42.A05(b)** to read as follows:

(b) *Final feed, 0.0075%.*—Weigh 20 g mash feed or pellets ground to pass No. 20 sieve and transfer to 500 ml vol. flask. Add 12 ml pH 4.7 buffer and wet feed thoroly. Immerse flask 5 min in 70° H_2O bath. Cool to room temp. Add 200 ml EtOAc, stopper, and shake mech. 10 min. Centrf. ca 100 ml EtOAc ext 10 min at

2000 rpm. Pipet 60 ml clear EtOAc ext into 200 ml vol. flask, add 8 ml 1.5*N* HCl, and shake 10 min. Let layers sep., transfer EtOAc layer to 100 ml g-s centrf. tube, and centrf. 10 min at 2000 rpm. Pipet 40 ml clear EtOAc ext into another 100 ml g-s centrf. tube and add 2 ml 40% NaOH soln. Stopper and shake briefly by hand, add 8 g anhyd. Na_2SO_4, and shake again. Centrf. 10 min at 2000 rpm and decant 25 ml clear supernate into 50 ml g-s graduate. Evap. all EtOAc under stream of N with graduate immersed in 60° H_2O bath. Dissolve residue in 5 ml hexane, add exactly 25 ml 75% MeOH (v/v), stopper, and shake vigorously 1 min. Transfer to 125 ml separator and let stand ca 1 hr. Withdraw lower (MeOH) layer into 25 ml beaker, pipet 5 ml into 50 ml vol. flask, and dil. to vol. with 19.4% (v/v) MeOH.

(e) Delete **42.A06.**

(f) Change **42.A07,** line 3 to read: "35±1°. Calc. L and H and fit" and delete lines 5–6.

Results and Discussion

Seven collaborators submitted results. Three (3, 6, 7) incubated the samples at 37°C instead of 35±1°C. Collaborator 7 modified the determination of the standard response line by placing the 5 standard dose levels on each plate and replicating this on 10 plates. Collaborator 5 separated the 75% methanol-hexane phases by centrifugation rather than by standing. Collaborator 3 submitted results for each mix in duplicate and it was apparent that a strong positive bias was affecting both sets of results. Part of the problem appeared to lie in a standard of low potency, but even after rechecking a new standard solution and correcting the values, the averages of the duplicate results lay outside the limits when tested according to Youden's rank sum test (3) for allowable approximate 5%, 2-tail limits. Therefore, the results of Collaborator 3 were omitted from the statistical calculations. Collaborator 5 had no difficulty with the A samples, but obtained low results for the B samples. A different lot of ethyl acetate was used for the B samples and the evaporation step in this second analysis took considerably longer than for the first analysis; it appeared that these factors could have affected the result. This collaborator ran out of nitrogen and was unable to procure more in time to finish the assays of the B samples. Repeat assays of the A samples by using rapid evaporation under a stream of air yielded results in close agreement with the results obtained with the nitrogen. Therefore, the B samples were reassayed using rapid evaporation with air. These results are listed in Table 1 for the B samples for Collaborator 5. Since 3 other collaborators also reported that they obtained similar results with air as with nitrogen, this substitution appears to be justified. Table 1 presents the results obtained for the mashes and pellets, including the average corrected data for Collaborator 3.

The average recoveries (Collaborator 3 excluded) for Mixes 1, 2, and 3 were: 97.5, 94.5, and 93.2%, respectively, for the mashes and 92.8, 87.8, and 87.8%, respectively, for the pellets. The corresponding coefficients of variation for the mash feeds were 12.3, 13.4, and 7.9% and for the pellets 11.1, 11.8, and 6.6%, respectively.

The data for the pairs of mash and pelleted feeds were evaluated statistically, using the procedure of Youden (3). The results are presented in Table 2. These statistical results include S_d (the overall standard deviation), S_r (the precision standard deviation), and S_b (the standard deviation for the distribution of systematic errors).

S_r values do not differ appreciably for the 3 pairs of mash feeds (4.61–6.74) and pelleted feeds (4.27–7.14). S_b is a direct measure of interlaboratory bias. The S_b/S_r ratios varied from 0.54 to 2.11 for the mashes and from 0.72 to 1.43 for the pelleted feeds.

Several collaborators reported that the method was straightforward, that no problems were encountered in the assay, and that the zones of inhibition were very good. One collaborator reported that the methodology was excellent. On the other hand, Collaborator 3 reported large variations in the size of zones of inhibition. The suggestion was made that at least 50 mg reference standard be weighed to prepare the stock standard dilution.

Four collaborators reported that evaporation with air yielded similar results to evaporation with nitrogen.

Recommendation

It is recommended that the modified microbiological assay method for lasalocid sodium in

Received August 15, 1975.

The recommendation of the Associate Referee was approved by the General Referee and Subcommittee G and was adopted by the Association. *See* (1976) *JAOAC* **59**, 398.

Table 1. Collaborative results for the microbiological determination of lasalocid sodium in feeds

Coll.	Mix 1 A	Mix 1 B	Mix 2 A	Mix 2 B	Mix 3 A	Mix 3 B
			Mash Feeds, ppm			
1	66	59	73	70	90	88
2	66	63	86	81	93	97
3[a]	78	89	91	101	135	112
4	71	80	94	99	97	107
5	58	54[b]	68	68[b]	100	85[b]
6	80	70	80	90	100	110
7	70	70	90	80	100	90
Av.	68.5	66.0	81.8	81.3	96.7	96.2
Av. dev.	5.17	7.33	8.17	8.77	3.43	8.5
Std dev.	7.26	9.27	10.05	11.79	4.27	10.94
Coeff. of var., %	10.6	14.0	12.3	14.5	4.4	11.4
Amt added	69	69	86.3	86.3	103.5	103.5
Rec., %	99.3	95.7	94.8	94.2	93.4	92.9
Lowest rec., %	84.0	78.3	78.8	78.8	87.0	82.1
Highest rec., %	115.9	115.9	108.3	117.0	96.6	106.3
			Pelleted Feeds, ppm			
1	59	54	67	64	88	81
2	65	63	80	77	87	95
3[a]	86	77	81	71	111	114
4	72	81	79	92	95	97
5	61	64[b]	77	72[b]	84	88[b]
6	60	70	70	70	90	95
7	60	60	90	70	100	90
Av.	62.8	65.3	77.2	74.2	90.7	91.0
Av. dev.	3.77	6.77	5.83	6.90	4.57	4.67
Std dev.	4.96	9.29	8.13	9.68	5.85	5.97
Coeff. of var., %	7.9	14.2	10.5	13.0	6.5	6.6
Amt added	69	69	86.3	86.3	103.5	103.5
Rec., %	91.0	94.6	89.5	86.0	87.6	87.9
Lowest rec., %	85.5	78.3	77.6	74.2	81.2	78.3
Highest rec., %	104.3	117.4	104.3	106.6	96.6	93.7

[a] Not included in statistical evaulation; *see* text.
[b] *See* text.

Table 2. Statistical evaluation of collaborative results for lasalocid sodium in feeds[a]

Statistic	Mash feeds Pair I	Mash feeds Pair II	Mash feeds Pair III	Pelleted feeds Pair I	Pelleted feeds Pair II	Pelleted feeds Pair III
S_d	10.81	14.64	8.47	9.63	10.20	6.64
S_r[b]	4.61	4.65	6.74	4.27	7.14	4.64
S_b	6.91	9.82	3.63	6.10	5.15	3.36
S_b/S_r	1.50	2.11	0.54	1.43	0.72	0.72
$(S_b^2 + S_r^2)^{1/2}$[c]	8.3	10.9	7.7	7.4	8.8	5.7

[a] Pair I—Mix 1, A and B; Pair II—Mix 2, A and B; Pair III—Mix 3, A and B.
[b] S_r is an estimate of expected within-laboratory variability or repeatability.
[c] $(S_b^2 + S_r^2)^{1/2}$ is an estimate of expected between-laboratory variability or reproducibility.

feeds, which involves changes in extraction procedure and highest level of standard, be adopted as official first action and that the study be continued.

Acknowledgments

Rosemarie Bertram, Ulric P. Green, and T. M. Kuervers, Agriculture Canada, Calgary, Alberta, Canada

Gordon G. Carter, Food and Drug Administration, Washington, DC

Jessica Chen, Hoffmann-La Roche Inc., Nutley, NJ

Raymond A. Davis and Gerald L. Stahl, The Upjohn Co., Kalamazoo, MI

Raymond A. Kubista, WARF Institute, Inc., Madison, WI

Dorothy L. Mueller, Ralston Purina Co., St. Louis, MO

H. S. Ragheb, Purdue University, West Lafayette, IN

This report of the Associate Referee was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

References

(1) Scheiner, J. (1975) *JAOAC* **58**, 941–944

(2) Osadca, M., & Araujo, M. (1975) *JAOAC* **58**, 507–512

(3) Youden, W. J. (1973) *Statistical Techniques for Collaborative Tests,* AOAC, Washington, DC

Rapid Colorimetric Method for Carbadox in Animal Feeds

VIRGINIA A. THORPE
Michigan Department of Agriculture, Laboratory Division, 1615 S Harrison Rd, East Lansing, MI 48823

The colorimetric method for carbadox in animal feeds was modified to be simpler and faster. Carbadox is extracted with dimethylformamide and separated from interferences on an alumina column in the absence of water by controlling the amount of sample extract on the column and the flow rate. The isolated carbadox is eluted into sodium hydroxide and the resultant yellow chromophor is measured spectrophotometrically at 420 nm.

The Goras *et al.* method (1) for carbadox, an antibacterial agent in swine feeds, involves extraction of the drug from the feed with chloroform-methanol (3+1), specific isolation of the carbadox from interferences in the feed matrix by a series of solvent-solvent extractions, evaporation of solvents, and reconstitution in dimethylformamide (DMF), followed by column chromatography. The isolated carbadox is extracted with sodium hydroxide to form a strong yellow chromophor which is measured spectrophotometrically at 420 nm. The identity is confirmed by the presence of a peak at about 350 nm.

Recoveries of carbadox from the feed matrix were about 88%. To compensate for this, the technique of standard additions was incorporated into the method. For every feed analyzed, 2 samples, with and without added standard, had to be analyzed also. Furthermore, pelleted feeds required a pretreatment with water to overcome an apparent drug binding problem.

We thought the method was basically sound; however, it seemed too involved, with many chances for error. The extraction step was long (1 hr), and maintaining a gentle boil for that length of time without undue loss of solvent was difficult. The solvent-solvent extractions sometimes developed hard-to-break emulsions and if traces of water were present after solvent evaporation, carbadox eluted in the DMF, preventing isolation of the carbadox on the alumina column.

The technique of standard addition is based on the assumption that identical recoveries are obtained for each sample. However, if lower recoveries are obtained for the sample with the added standard, results are positively biased, and high results have been our experience with this technique.

In 1975, the above method was modified and subjected to a collaborative study (J. T. Goras, 1975, Pfizer Inc., Groton, CT). The color developed for carbadox was more specific, thus eliminating the column cleanup step but retaining the water pretreatment step, method of standard additions, long extraction time, solvent-solvent extractions, evaporations, and a more complex color development.

Our laboratory made further modifications to create a simple, short, and precise method that appears worthy of further study.

METHOD

(*Caution:* Solutions of carbadox are light-sensitive. Protect feed and supplement extracts from direct sunlight or artificial light.)

Apparatus

(a) *Spectrophotometer.*—Beckman Model 24/25, or equivalent.

(b) *Chromatographic tubes.*—Kimflow 28580, size 10-C, or equivalent.

(c) *Centrifuge.*—International clinical centrifuge with head to accommodate 50 ml centrifuge tubes, or equivalent.

Reagents

(a) *Alumina.*—Alcoa F-20, 80–200 mesh (Fisher Scientific Co., A-540). If carbadox is not retained on adsorbent, activate 18 hr at 200°C.

(b) *Carbadox standard solutions.*—(Obtain reference standard from Pfizer Agricultural Division, Lee's Summitt, MO 64063.) (*1*) *Stock solution.*—1.10 mg/ml. Weigh 110 mg carbadox reference standard into 100 ml volumetric flask, dilute to volume with $CHCl_3$-methanol (3+1), and mix well. (*2*) *Working solution.*—0.11 mg/ml. Pipet 10 ml stock solution into 100 ml volumetric flask, dilute to volume with $CHCl_3$-methanol (3+1), and mix well. (*3*) *Working solution.*—0.022 mg/ml. Pipet 5 ml stock solution into 250 ml volumetric

flask, dilute to volume with $CHCl_3$-methanol (3+1), and mix well. Standards are stable up to 1 year if stoppered tightly and protected from light.

Preparation of Standards

Prepare 10-fold dilutions of working standards to obtain standard solutions containing 2.2 and 11.0 μg carbadox/ml $CHCl_3$-methanol (3+1).

(a) *Standard for 0.0011% carbadox.*—Pipet 10.0 ml 2.2 μg/ml standard solution into 25 ml glass-stoppered graduate. Add 5 ml 0.1*N* NaOH and 5 ml $CHCl_3$-methanol (3+1). Invert cylinder twice and let settle in dark until bottom and top menisci are clear. Measure volume of NaOH layer, estimating to nearest 0.1 ml. Remove NaOH layer, centrifuge, and read absorbance at 420 nm against water. Convert absorbance reading equivalent to exactly 5 ml volume of 0.1*N* NaOH.

(b) *Standard for other concentrations.*—0.0055, 0.011, 0.022, 0.055%, etc. Pipet exactly 10 ml 11.0 μg/ml standard solution into 50 ml glass-stoppered graduate. Add 20 ml 0.1*N* NaOH and 10 ml $CHCl_3$-methanol (3+1). Invert cylinder twice and proceed as above, beginning ". . . let settle . . .". Convert absorbance reading equivalent to exactly 20 ml volume of 0.1*N* NaOH.

Preparation of Column

Add 10 g alumina to chromatographic tube. Tap once gently. Column must not be packed tightly. Add 10 ml DMF; let drain until 1 ml remains above bed level. Column is ready for aliquot of sample extract. Check that flow rate is 1.5–2.5 ml/min. If too slow, replace fritted disk or repack column. If too rapid, repack column because carbadox will not be adsorbed.

Preparation of Sample Extract

(*Note:* Glassware must be free of water. Traces of water will cause carbadox to elute from column with DMF.)

Grind coarse or pelleted feeds to pass 20 mesh sieve. For guarantees of 0.0011–0.0055%, weigh 10 g samples; for >0.0055–0.011%, weigh 5 g samples; for 0.022%, weigh 2.5 g samples; and for 0.055%, weigh 1.0 g samples. Place in 125 ml glass-stoppered Erlenmeyer flask. Add 50.0 ml DMF, stopper loosely, and place in boiling water bath 5 min, shake mechanically 10 min, and filter through rapid paper.

Determination

Pipet 10.0 ml aliquot of sample extract onto prepared column and let pass through column by gravity. Wash column with 2 and 8 ml increments of DMF. Additional washing with DMF may be necessary if carotenoid pigments do not elute completely. If sample contains high levels (>2.5%) of alfalfa meal, wash until eluate is pale green. Eliminate DMF from column by washing with 20–25 ml absolute ethyl ether, using gentle suction until ether is removed. Discard washings. Elute carbadox with 20 ml $CHCl_3$-methanol (3+1) (15 ml for guarantees of 0.0011%). Collect eluate containing carbadox in 50 ml glass-stoppered graduate containing 20 ml 0.1*N* NaOH (25 ml glass-stoppered graduate containing 5.0 ml 0.1*N* NaOH for guarantees of 0.0011%). Invert cylinder twice and proceed as in *Preparation of Standards,* beginning ". . . let settle . . .". Convert absorbance reading equivalent to exactly 20 ml and/or 5 ml volume of 0.1*N* NaOH.

$$\text{Carbadox, } \% = (A/5 \text{ ml} \times 0.0011\%)/A'/5 \text{ ml}$$

$$\text{Carbadox, } \% = (A/20 \text{ ml} \times 0.005\%)/A'/20 \text{ ml}$$

where A and A' = absorbance at 420 nm of sample and standard, respectively.

Discussion

It was noted in our laboratory that carbadox interfered in the furazolidone method, AOAC 42.069 (2), and further study showed that both have similar chemical properties. Carbadox is soluble in DMF when the solution is warmed. Furazolidone is soluble in chloroform-methanol (3+1), but not as soluble as carbadox. DMF solutions of both have the same absorption characteristics on alumina. Both form chromophores with sodium hydroxide, although carbadox forms a more intense color than furazolidone. Both form phenylhydrazone derivatives, but carbadox forms a less intense color than furazolidone. Carbadox can be determined by the official furazolidone method (42.069 (2)); results are identical to those for the rapid method described, but the method is less sensitive and involves more steps. With this background, we thought that carbadox could be extracted in the same manner as furazolidone. If water were absent, then carbadox could be separated from feed interferences by column chromatography on basic alumina. If it could be specifically isolated and eluted, then the simple and more sensitive yellow chromaphor formed with sodium hydroxide could be used for the final measurement.

Received April 1, 1976.

Carbadox in feed exhibited the same solubility in DMF when placed in a boiling water bath for 5 min and mechanically shaken for 10 min as when boiled with chloroform-methanol (3+1) for 1 hr. Solubility data were obtained by using a sample with a guarantee of 0.0055% carbadox. A 20 g sample was extracted with 140 ml chloroform-methanol (3+1) according to the collaborative method (1) and diluted to 250 ml, a 50 ml aliquot was evaporated to dryness and dissolved in 20 ml DMF, and a 10 ml aliquot of DMF solution was carried through the rapid method simultaneously with a sample dissolved in DMF according to the rapid method. To determine if losses occurred during the evaporation step, standards of the same concentration and in the same volume of solvent were carried through the evaporation step and compared with standards without evaporation. Measurements with and without evaporation were the same; therefore, no losses occurred during evaporation. Identical results were obtained for the different solubility techniques. Carbadox was also determined by the official furazolidone method (**42.069** (2)) with chloroform-methanol (3+1) or DMF, and no differences were detected.

Several brands of basic alumina prepared under different conditions were tested to determine which one retained a DMF solution of carbadox in the presence of interferences extracted from feed. Alcoa F-20, 80–100 mesh, met the requirements without prior treatment.

Conditions affecting the absorption were the amount of feed extract placed on the column, the flow rate of the column, and the presence of water. The limits of these variables were established and sample weights, volume of extract, and aliquots placed on the column were specified. The flow rate was controlled by column packing and a fritted disk (a glass wool plug and stopcock could also be used). Glassware that was not free of moisture was oven-dried.

When these conditions were met, we were able to extract the carbadox from the feed with DMF, specifically isolate and elute carbadox from all interfering substances, and use the simple, sensitive sodium hydroxide chromophor for final measurement. By eluting directly into a glass-stoppered graduate and measuring the resultant volume of sodium hydroxide, we simplified the procedure and still maintained the necessary parameters on the column at the 0.0011% level.

Pretreatment with water for pelleted feeds was impossible because water will carry through and elute carbadox in the DMF solution. No means have been found for drying the DMF solution. Analyses performed in the Associate Referee's laboratory (1) showed a consistent increase in results after water treatment. Without water treatment, recoveries were about 94%. This recovery factor could be inserted in the calculations for pelleted feeds if, in fact, a drug binding problem does exist. No mention of this has been made with furazolidone, a drug with similar properties that is extracted in the same manner. Our laboratory cannot formulate medicated feeds, so we have no means of checking this problem.

Recoveries were studied by using samples submitted for the 1976 collaborative study of Goras *et al.* (unpublished (1975)), with guarantees of 0.0011, 0.0055, and 0.022%. Five ml working standard (0.022 mg/ml for 0.0011% level and 0.110 mg/ml for 0.0055 and 0.022% levels) was placed in a 125 ml glass-stoppered Erlenmeyer flask and evaporated to dryness. The appropriate size sample was then placed in the flask containing the evaporated standard. This sample plus standard and an identical sample without added standard were analyzed. Results were compared with standards analyzed directly as under *Preparation of Standards*. In all cases, 100% recoveries were obtained. Recoveries of added standards in the 1976 collaborative study of Goras *et al.* averaged 88.3% in our laboratory.

In studying the chromatographic step of the method, it is important to adhere to the sample weights and aliquots as specified in the method. When the aliquot of the sample extract has passed through the column, the carotenoid pigments are completely eluted with a total of 10 ml DMF. Additional washing may be necessary when feeds are formulated with high (>2.5%) levels of alfalfa meal. Washing with DMF is continued until the eluate is pale green. When the carbadox is eluted with chloroform-methanol (3+1) and extracted with NaOH, the pale green color remains in the chloroform fraction. Feeds formulated in the laboratory with up to 25% alfalfa meal showed no interference in the rapid carbadox method. Most swine feeds are formu-

Table 1. Comparison of results for 6 collaborative samples analyzed by 1976 collaborative method and proposed rapid method

Intended potency	Carbadox, %				Recovery, %	
	Coll.	Av.	Rapid	Av.	Coll.	Rapid
0.00132	0.00112	0.00116	0.00100	0.00096	87.8	72.7
	0.00120		0.00092			
0.00132	0.00117	0.00124	0.00126	0.00125	93.9	94.7
	0.00131		0.00123			
0.00529	0.00558	0.00573	0.00469	0.00475	108.3	89.8
	0.00587		0.00480			
0.00529	0.00535	0.00538	0.00471	0.00478	101.7	90.4
	0.00541		0.00484			
0.0242	0.0244	0.0255	0.0224	0.0229	105.4	94.6
	0.0266		0.0234			
0.0242	0.0286	0.0264	0.0237	0.0236	109.1	97.5
	0.0242		0.0234			

lated with not more than 2.5% alfalfa meal, and at this level, additional washing is unnecessary.

Using the samples submitted for the 1976 collaborative study, results were obtained by the rapid method and compared with results obtained by the method under study. The rapid method results were highly reproducible; 6 samples can be completed in 2 hr without experience or special techniques. The long method, with experience and more careful techniques, would require several days for the analysis of 6 samples. Results by the rapid method tend to be low and results by the collaborative method tend to be high (*see* Table 1). We know carbadox can be extracted with DMF (5 min in a boiling water bath and shaking mechanically 10 min), as well as with chloroform-methanol (3+1) (boiling for 1 hr). We know recoveries of standards added to samples are consistently 100%. After data were obtained, we learned that the first 4 samples used in the collaborative study were pelleted feeds and we were also informed of the intended potency of each sample. With the exception of Sample 1, recoveries by the rapid method ranged from 89.8 to 97.5%. One pelleted feed and one feed not pelleted gave recoveries of 94.7 and 94.6%, respectively. Drug binding is not indicated.

The rapid method appears to be worthy of further study.

References

(1) Goras, J. T., Gonci, D. A., Kotaro, M., Curley, J. E., & Gordon, P. M. (1974) *JAOAC* **57**, 982–986

(2) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC

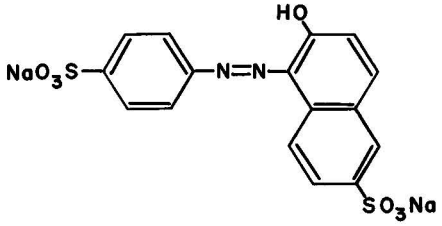
COLOR ADDITIVES

Preparation and Spectral Compilation of FD&C Red No. 40 Intermediates and Subsidiary Dyes[1]

SANDRA J. BELL
Division of Color Technology, Food and Drug Administration, Washington, DC 20204

FD&C Red No. 40 may contain higher or lower sulfonated subsidiary dyes, depending on the purity of intermediates used. Samples of 10 possible subsidiaries were prepared and purified. They are to serve as analytical standards to aid in the identification of unknown compounds frequently appearing during the analysis of certification samples. Visible, ultraviolet, and infrared spectra as well as absorptivities and molar extinction coefficients are given for each dye.

FD&C Red No. 40, 6-hydroxy-5-(2-methoxy-5-methyl-4-sulfophenylazo)-2-naphthalene sulfonic acid disodium salt is listed in the *Code of Regulations* (1) for provisional use in foods, drugs, and cosmetics. The amounts of higher and lower sulfonated subsidiary dyes permitted in certified batches of FD&C Red No. 40 are limited to 1% each. The dye is prepared by coupling diazotized cresidine-*p*-sulfonic acid (CSA) with 2-naphthol-6-sulfonic acid sodium salt (Schaeffer's salt). Technical grade intermediates, which are used in the commercial manufacture of dyes, are known to contain impurities which can either diazotize or couple to give subsidiary dyes. Subsidiary dyes can most likely arise from the presence of impurities such as R-salt (2-naphthol-3,6-disulfonic acid), G-salt (2-naphthol-6,8-disulfonic acid), 2-naphthol, or 2-naphthol-3,6,8-trisulfonic acid (NTA) in the Schaeffer's salt. They can also originate from the presence of cresidine or *o*-anisidine-*p*-sulfonic acid (OAS) in CSA. Ten subsidiary dyes have been prepared by using various combinations of these possible impurities.



Methods for the diazotization of CSA, cresidine, and OAS are given below. The general coupling method, used to form the desired subsidiary dyes, was not adequate for the formation of G-salt subsidiaries, since a mixture which contains very little of the desired product is obtained. A separate method is presented for the coupling of G-salt intermediates.

Experimental

Apparatus

(a) *Thin layer chromatographic apparatus.*—8 × 8″ thin layer plates coated with 250 μm silica gel G and developing tank large enough to accommodate plate.

(b) *High-pressure liquid chromatograph.*—Du Pont Model 830 with 1 m Zipax column (strong anion exchange) and variable wavelength detector.

(c) *Carbon-hydrogen-nitrogen analyzer.* — Perkin-Elmer Model 240.

(d) *Atomic absorption apparatus.*—Jarrell-Ash Model 82-385, for sodium and potassium determinations.

Diazotization

CSA or OAS.—Add 300 ml water and 5.9 g sodium carbonate to 0.1 mole CSA or OAS, and stir until dissolved. Place in ice bath, and stir until temperature equilibrates at 0°C. Add 7 g sodium nitrite and 50 g ice, and stir; quickly add 21 ml concentrated HCl, and stir additional 20 min. Test for excess nitrite with starch-iodide paper. If test is positive, add dropwise cold solution of 10% sulfamic acid until negative test is obtained.

Cresidine.—Cresidine is not water soluble. Add 300 ml water and 21 ml concentrated HCl to 0.1 mole cresidine to solubilize. Cool to 0°C; then add cold solution of 7 g sodium nitrite/30 ml water. Stir; then proceed as above with CSA.

Coupling

General method.—Add 150 ml water to 0.1 mole R-salt, Schaeffer's salt, etc. Add 10 g NaOH, and

[1] *See* (1975) *JAOAC* **58**, 1087–1128 for similar data for FD&C Yellow No. 6.

stir until solution is complete. Dissolve unsulfonated cresidine in aqueous HCl. Cool intermediate solutions to 0°C, and gradually add diazotized solution. Stir ca ½ hr; then neutralize with HCl. Add NaCl and continue to stir ½ hr to precipitate dye. Reduce solution volume with air if dye does not precipitate easily. Filter dye, re-dissolve in minimum amount of water, and re-precipitate with salt. Repeat again to eliminate remaining intermediates.

G-salt coupling.—Dissolve G-salt in 50% aqueous pyridine containing 10 g NaOH, cool to 0°C, and add diazotized component slowly and with stirring. Precipitate dye by adding acetone-ethyl ether until one phase forms. Re-dissolve dye, and precipitate with sodium acetate. Filter; then repeat salting-out process twice.

Purification

Purified samples of G-salt, R-salt, and 2-naphthol were available for use as starting material in the syntheses. Other intermediates and the subsidiary dyes prepared from them were further purified as given.

Intermediates

CSA.—Slurry 100 g CSA with 100 ml water, and add 50% NaOH solution until clear solution results. Add 20 g decolorizing carbon and heat slurry on steam bath ½ hr. Filter off carbon; then acidify with HCl. Filter precipitated CSA, wash with ethanol, and dry.

Cresidine.—Slurry 200 g cresidine with 500 ml hexane. Heat on steam bath until dissolved. Let solution cool slowly to room temperature. Filter and discard first batch of crystals. Filter and dry subsequent batches.

Schaeffer's salt.—Slurry 50 g Schaeffer's salt with 300 ml water. Make alkaline with NaOH; then heat ½ hr with 10 g decolorizing carbon. Filter; add HCl until intermediate begins to precipitate. Let cool, and filter.

NTA.—Dissolve 50 g in water, add 10 g decolorizing carbon, and heat ½ hr. Filter, add ethanol to precipitate intermediate, and filter.

OAS.—Heat to dissolve 50 g in minimum amount of methanol. Filter; then set in ice bath to precipitate product. Filter.

Subsidiary Dyes

All dyes are salted out several times as given above. Upon further recrystallization, consecutive precipitates are obtained for each. They were kept separate until purity was established by carbon-hydrogen-nitrogen analyses and by high-pressure liquid chromatography (HPLC). Impure fractions were discarded. Those showing the best analyses were combined; the recrystallization steps were repeated until adequate purity was obtained.

Cresidine + 2-naphthol.—Dissolve in minimum amount of acetone, adding solvent gradually while heating, until dye is in solution. Filter hot; then let cool to room temperature. Filter precipitate. Obtain further precipitates by cooling in ice bath and then placing in refrigerator overnight.

Cresidine + cresidine.—Heat to dissolve in minimum amount of acetone. Filter; add water until dye begins to precipitate. Re-heat; then cool in ice bath. Filter precipitate. Recrystallize again by dissolving in acetone and adding hexane to obtain precipitates.

CSA + 2-naphthol and cresidine + Schaeffer's salt.—Heat to dissolve dye in minimum amount of ethanol, filter, and place in refrigerator overnight. Filter precipitate, and obtain subsequent precipitates by adding small amounts of ethyl ether.

OAS + Schaeffer's salt.—Heat to dissolve dye in minimum amount of methanol. Filter; then add small amounts of hot acetone until dye begins to precipitate. Cool in ice bath, and filter precipitate. Obtain additional products by adding small quantities of acetone, heating, and then cooling in ice bath.

Cresidine + G-salt and cresidine + R-salt.—Heat to dissolve dye in minimum amount of methanol. Filter; then add hot ethanol until dye begins to precipitate. Cool in ice bath, and filter product. Obtain further precipitates by adding small amounts of ethanol, heating to redissolve, and then cooling overnight in refrigerator.

CSA + NTA.—Dye does not precipitate from reaction mixture with salt. Reduce volume by heating on steam bath; then add acetone-ethyl ether until one phase forms and dye begins to precipitate. Place in refrigerator overnight; then filter product.

CSA + G-salt.—Heat to dissolve in minimum amount of water, filter, and add hot ethanol until dye begins to precipitate. Cool in refrigerator overnight. Filter product, and add ethanol to obtain additional precipitates. Repeat, using water-ethanol.

CSA + R-salt.—Heat to dissolve in minimum amount of water, and precipitate with concentrated HCl. Recrystallize from water-methanol as above for CSA + G-salt.

Establishment of Purity

All subsidiary dyes were dried overnight in a vacuum oven at 110°C. They were allowed to equilibrate with atmospheric humidity in order to

Received October 6, 1975.

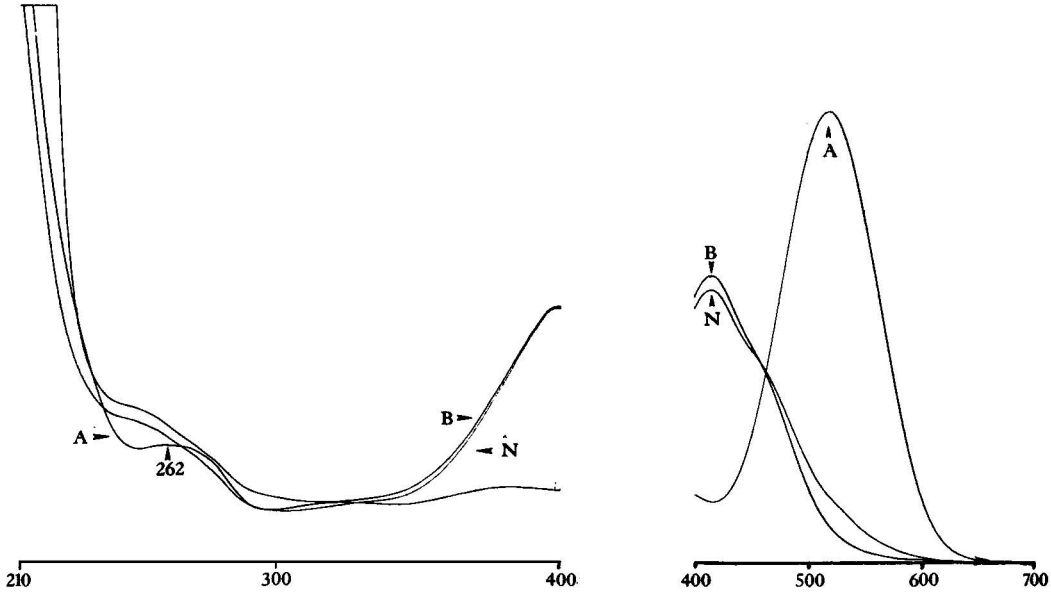


FIG. 1—UV-VIS spectra of cresidine + cresidine.

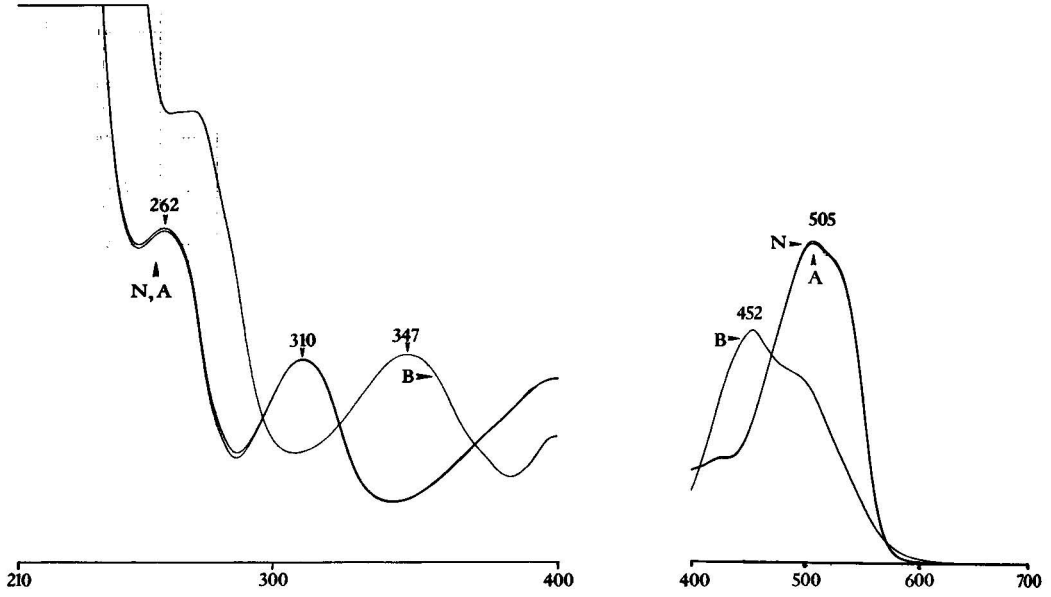


FIG. 2—UV-VIS spectra of cresidine + 2-naphthol.

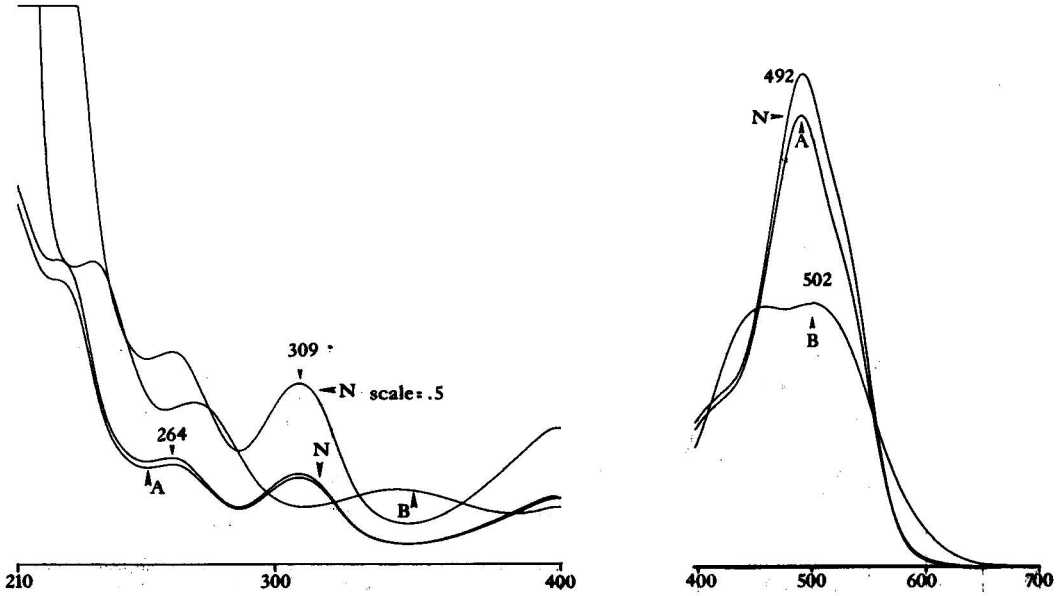


FIG. 3—UV-VIS spectra of CSA + 2-naphthol.

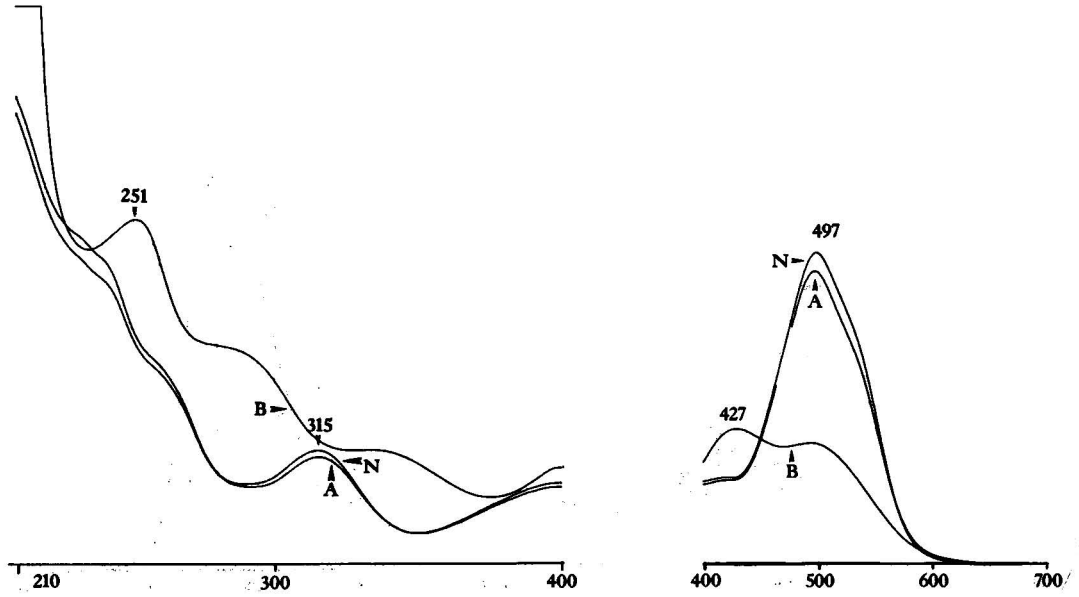


FIG. 4—UV-VIS spectra of cresidine + Schaeffer's salt.

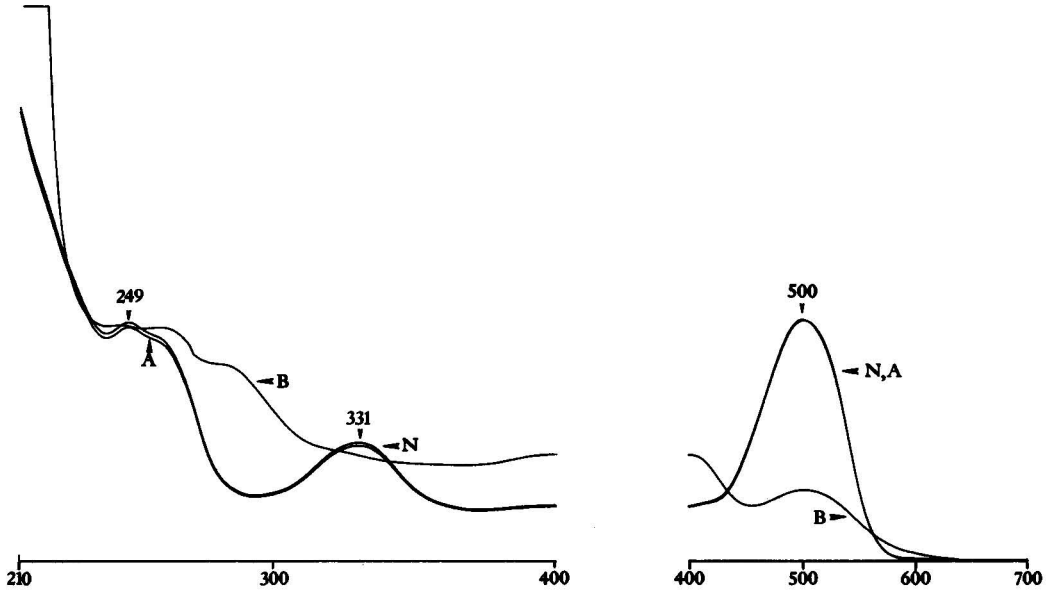


FIG. 5—UV-VIS spectra of cresidine + G-salt.

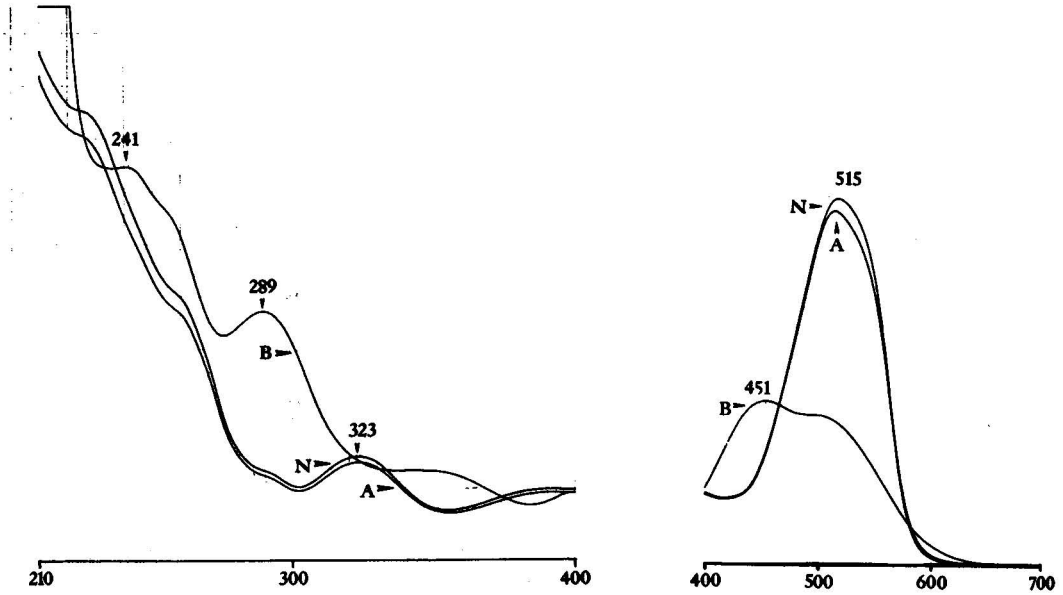


FIG. 6—UV-VIS spectra of cresidine + R-salt.

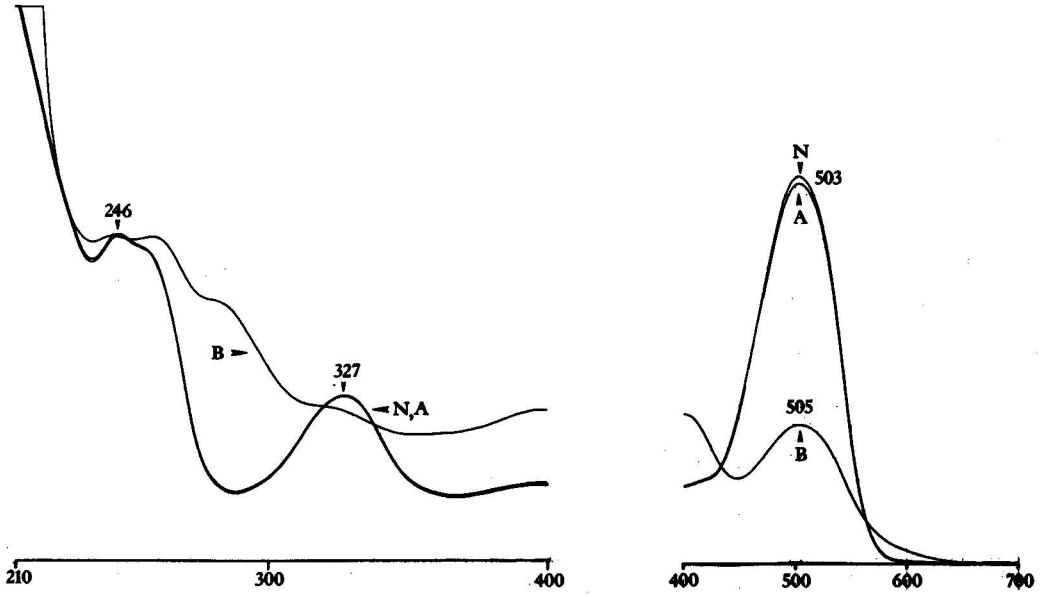


FIG. 7—UV-VIS spectra of CSA + G-salt.

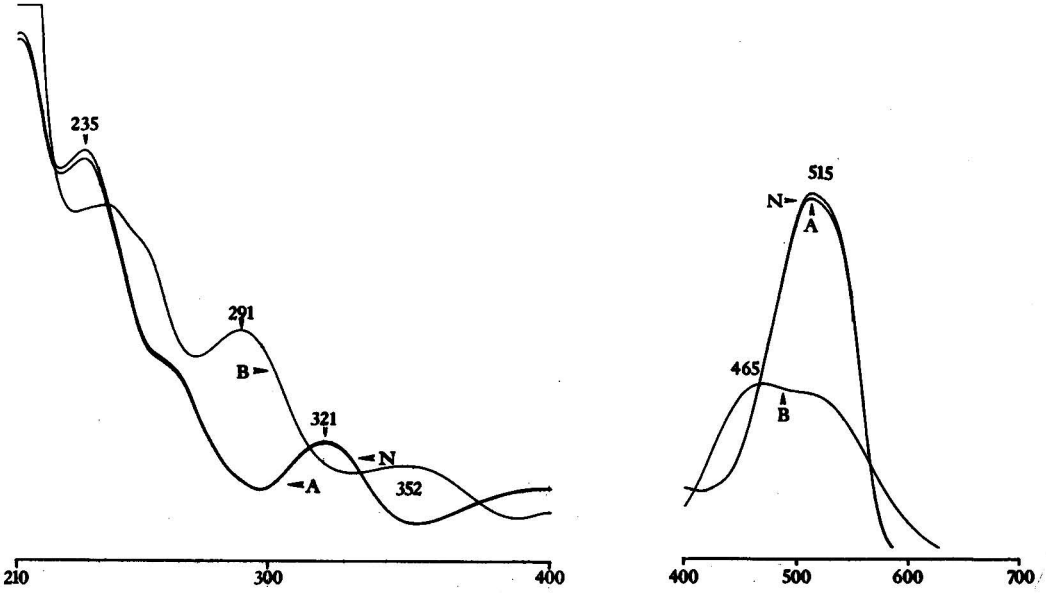


FIG. 8—UV-VIS spectra of CSA + R-salt.

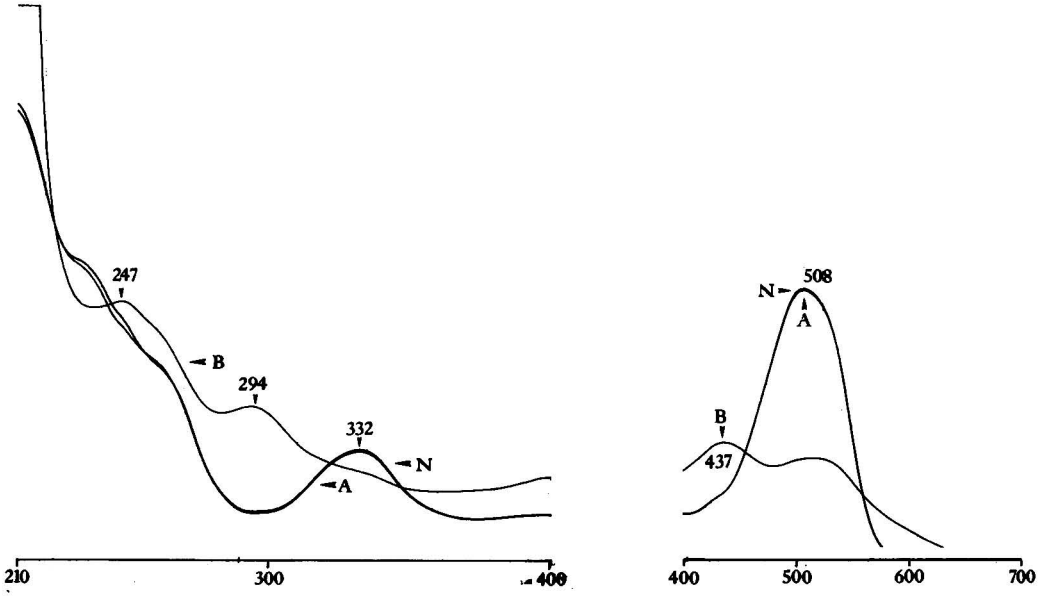


FIG. 9—UV-VIS spectra of CSA + NTA.

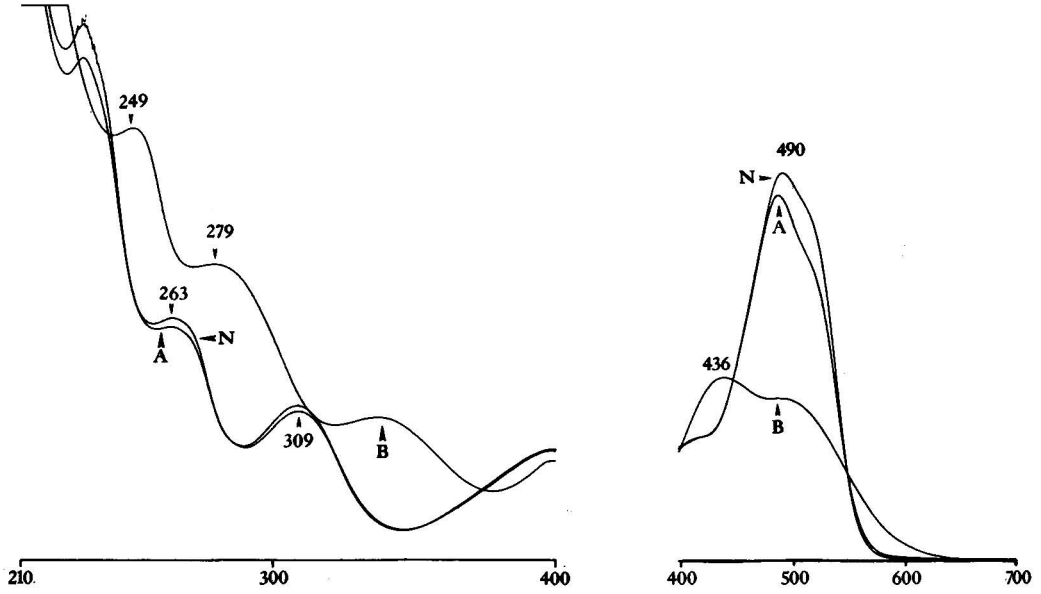


FIG. 10—UV-VIS spectra of OAS + Schaeffer's salt.

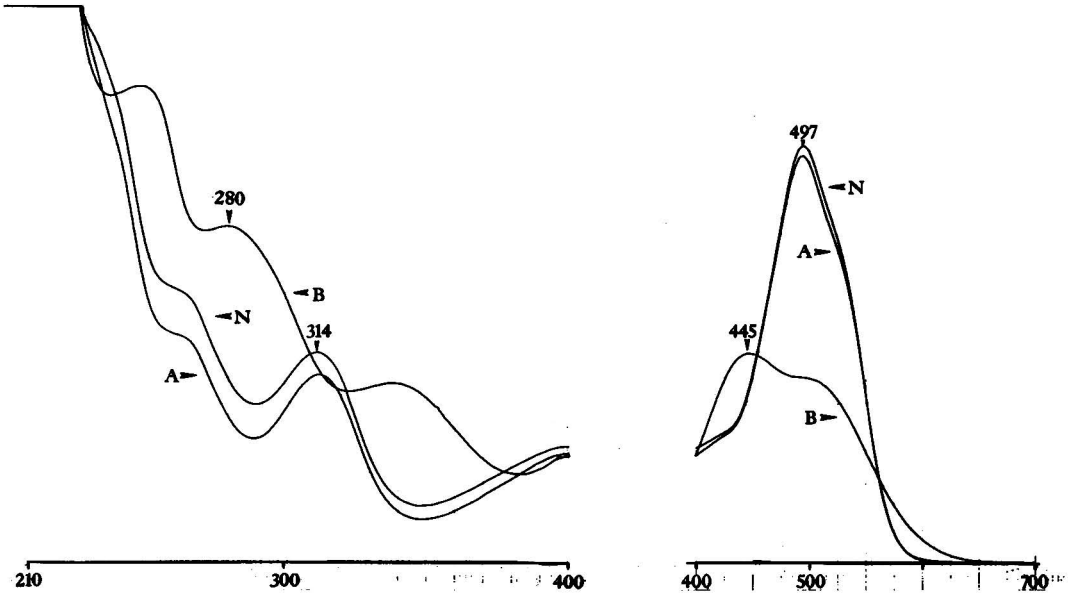


FIG. 11—UV-VIS spectra of FD&C Red No. 40.

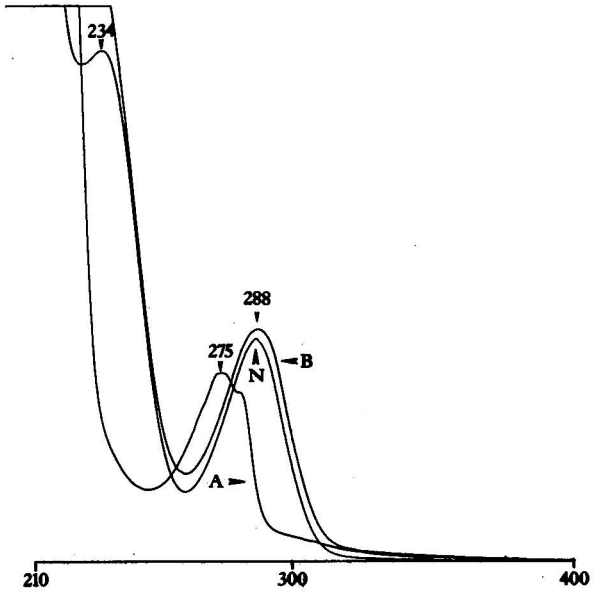


FIG. 12—UV spectrum of cresidine.

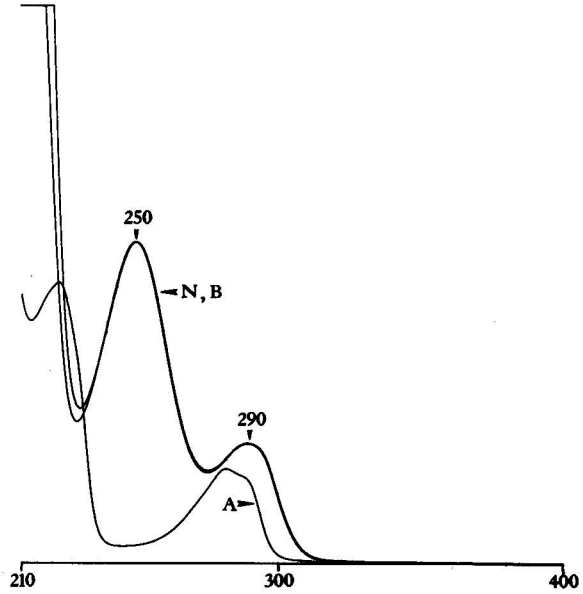


FIG. 13—UV spectrum of CSA.

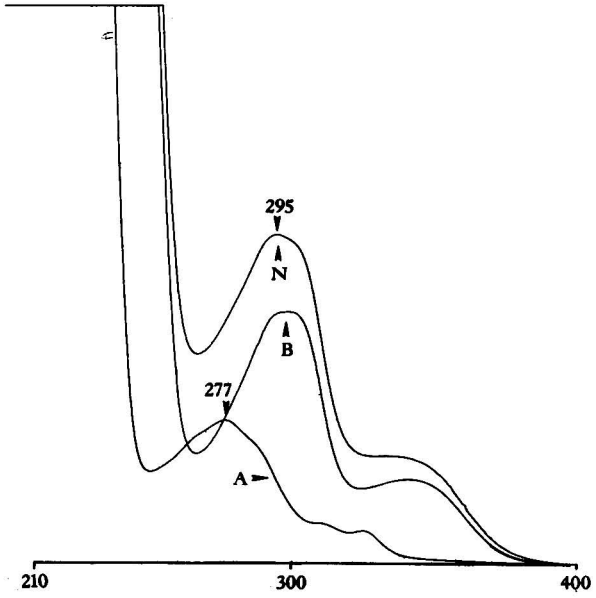


FIG. 14—UV spectrum of Schaeffer's salt.

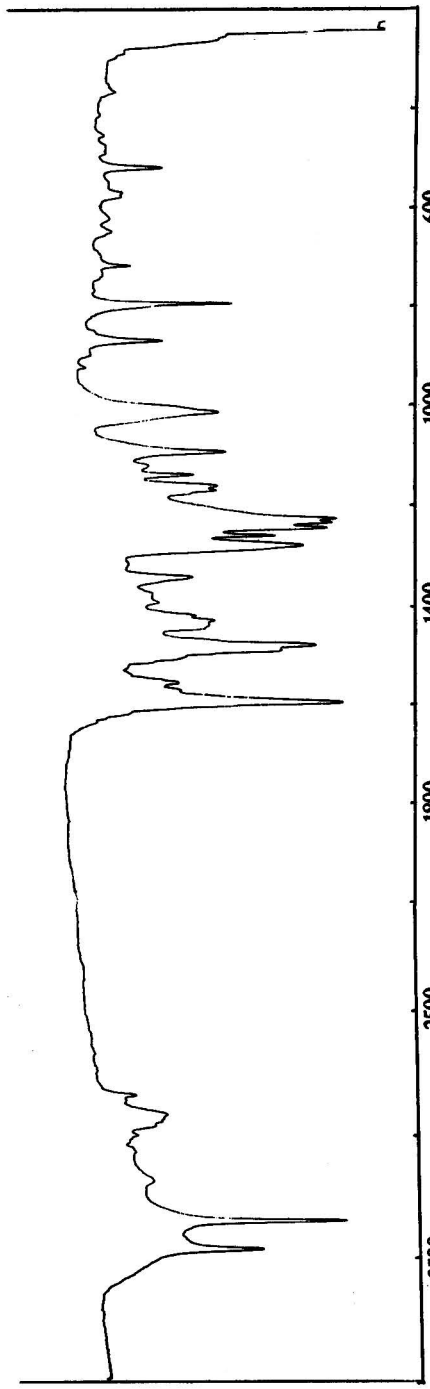


FIG. 15—IR spectrum of cresidine + cresidine.

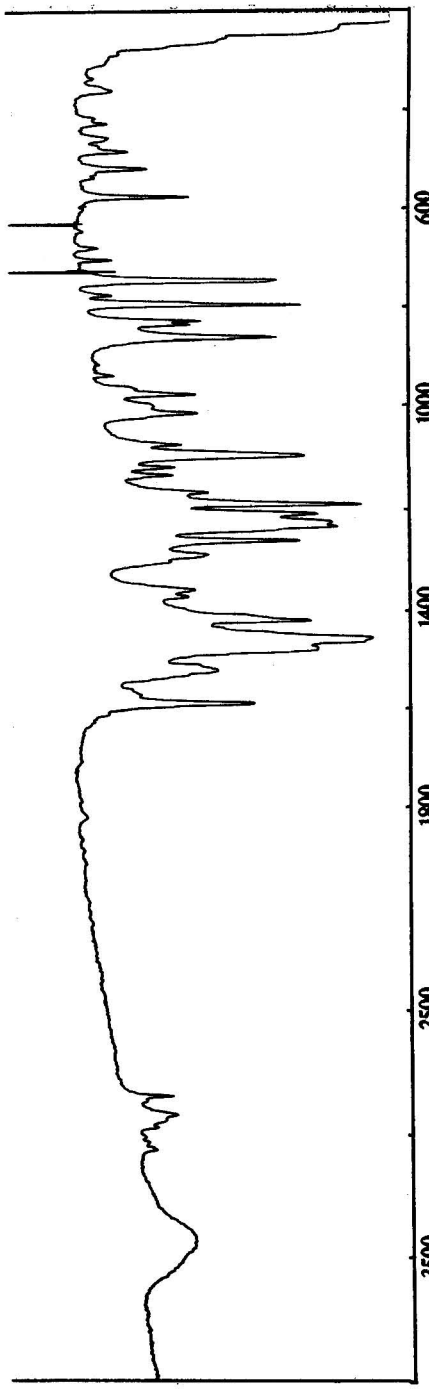


FIG. 16—IR spectrum of cresidine + 2-naphthol.

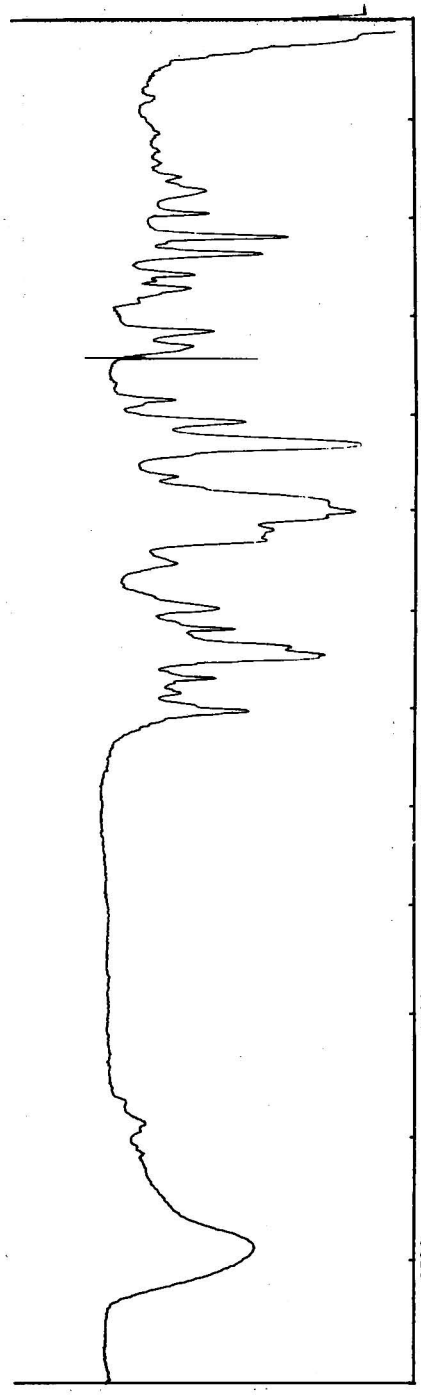


FIG. 17—IR spectrum of CSA + 2-naphthol.

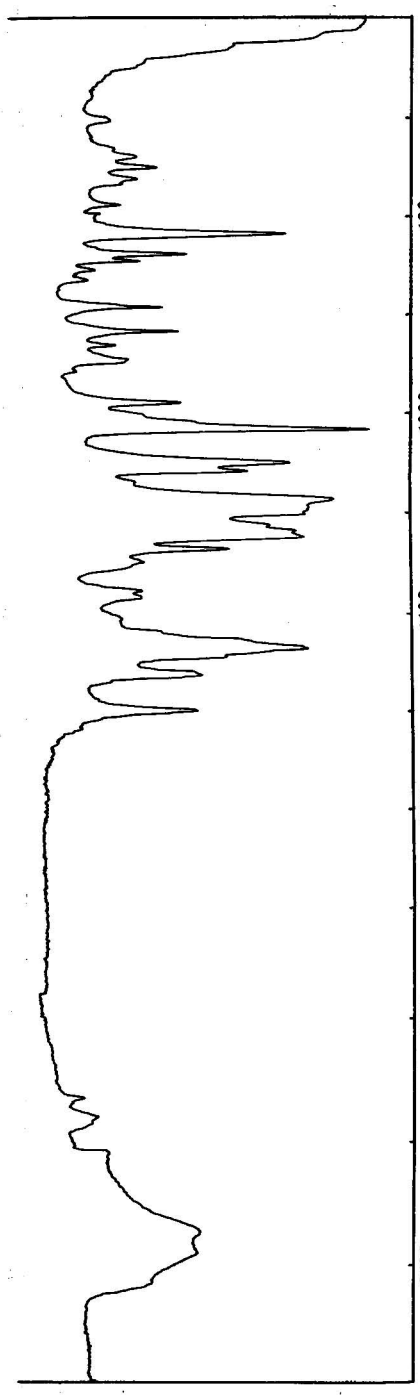


FIG. 18—IR spectrum of cresidine + Schaeffer's salt.

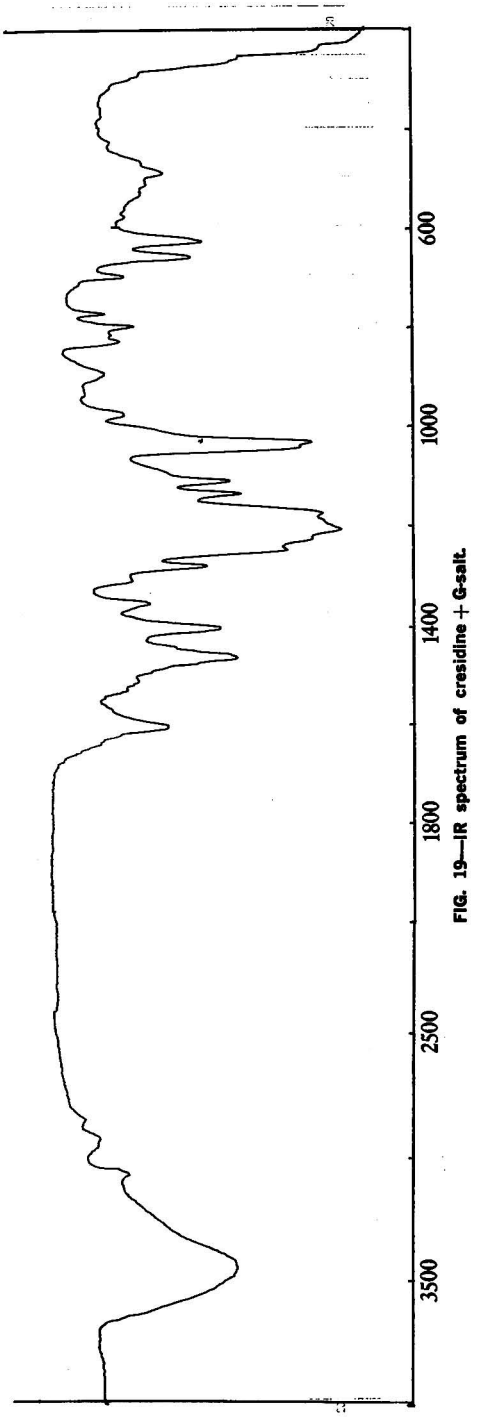


FIG. 19—IR spectrum of cresidine + G-salt.

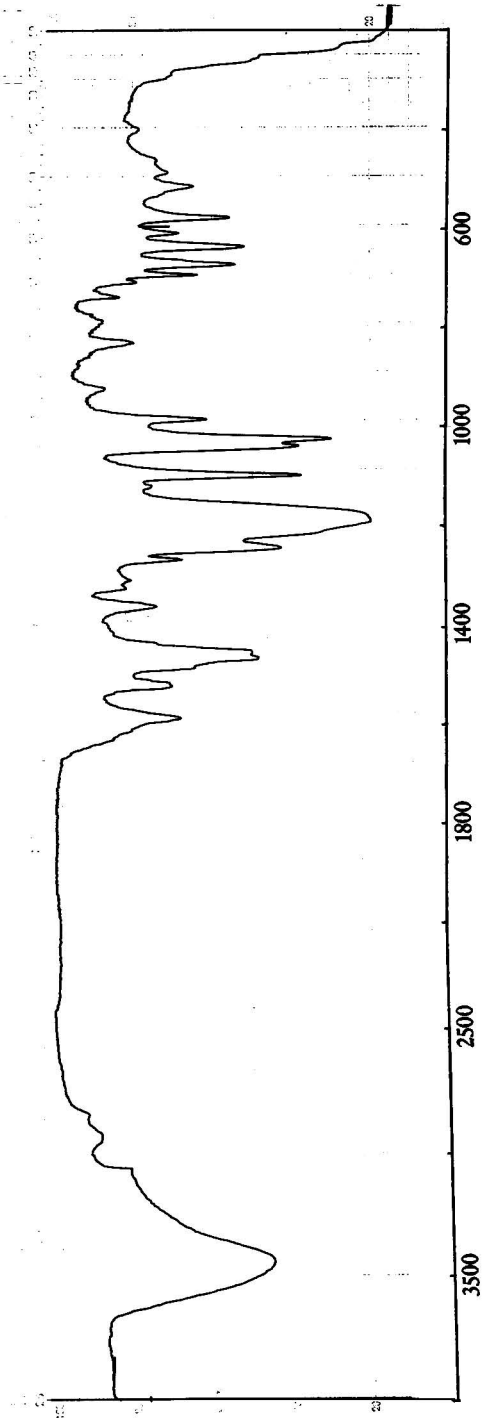


FIG. 20—IR spectrum of cresidine + R-salt.

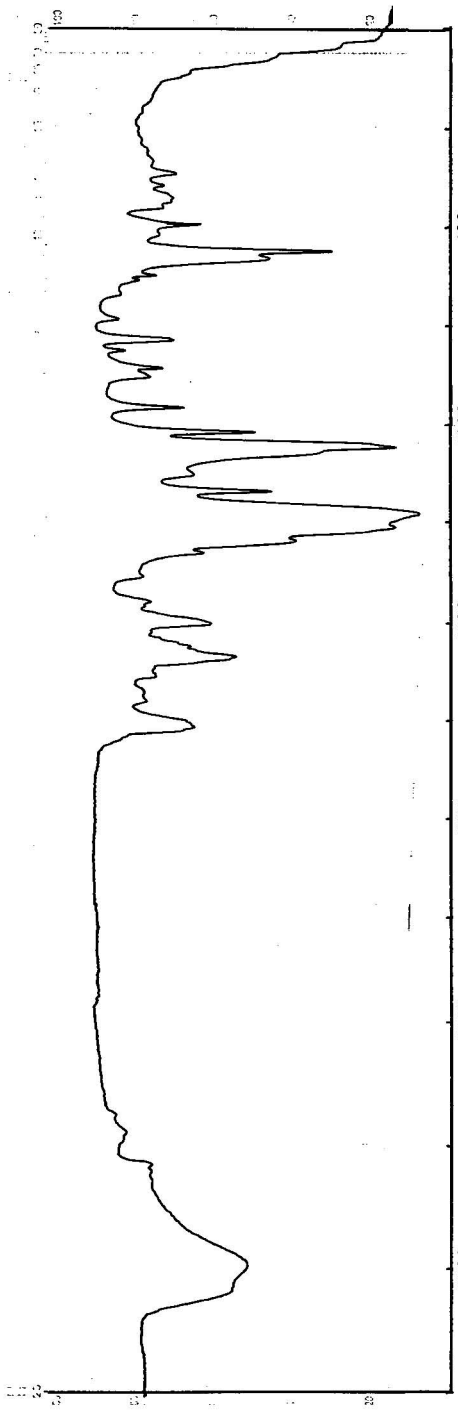


FIG. 21—IR spectrum of CSA + G-salt.

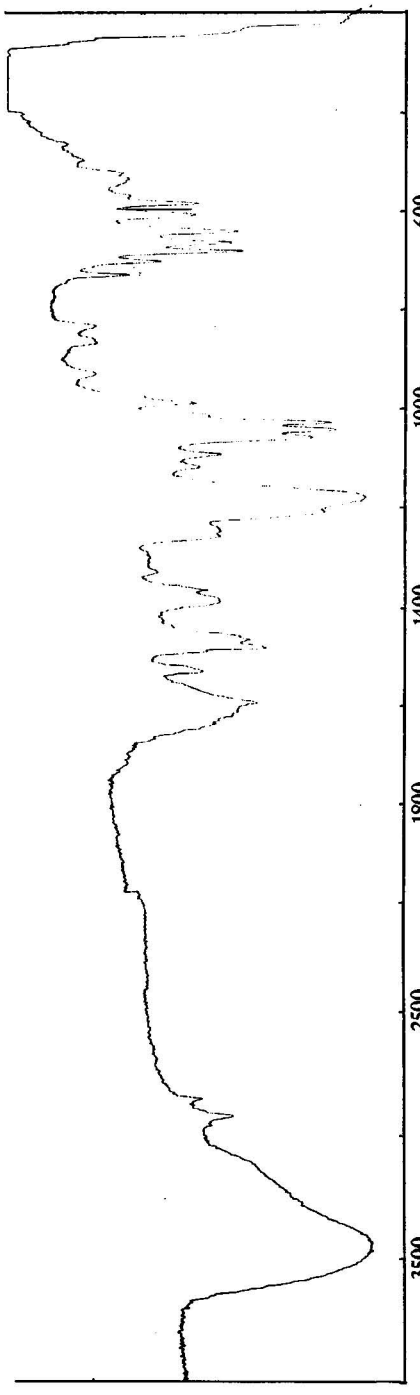


FIG. 22—IR spectrum of CSA + R-salt.

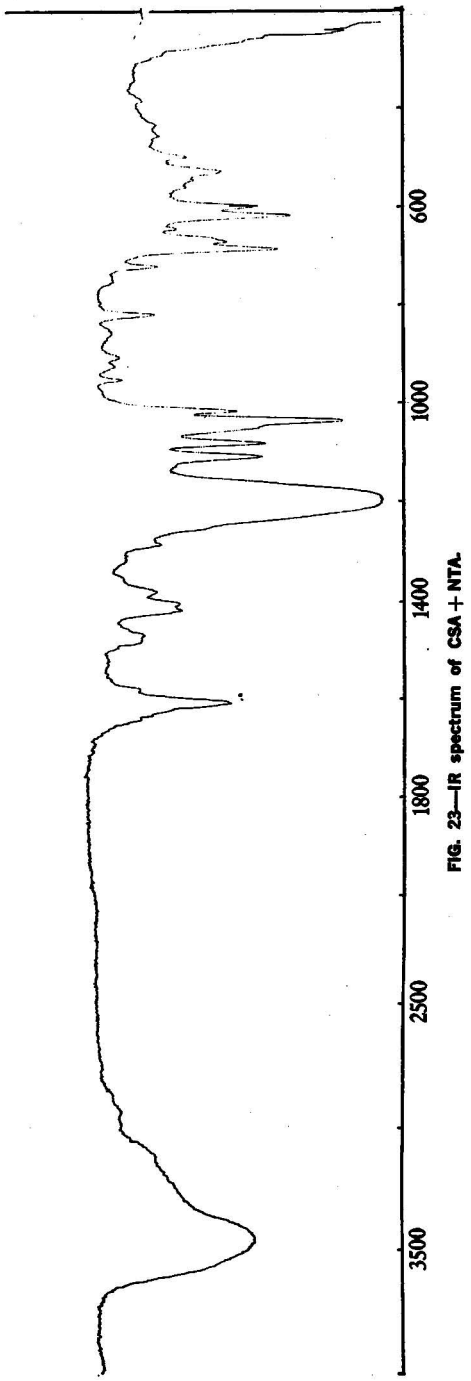


FIG. 23—IR spectrum of CSA + NTA.

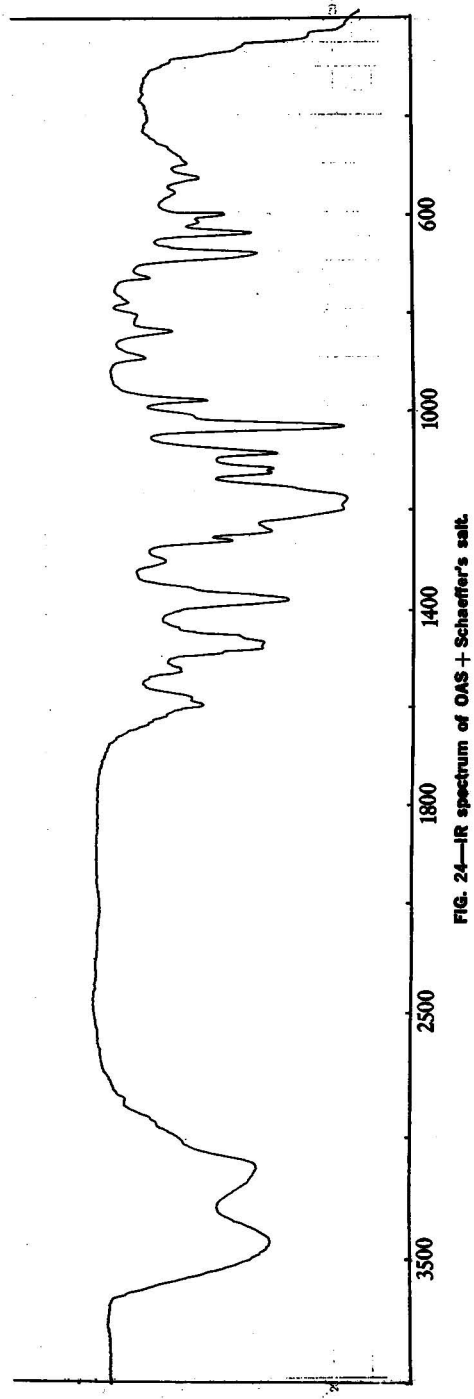


FIG. 24—IR spectrum of OAS + Schaeffer's salt.

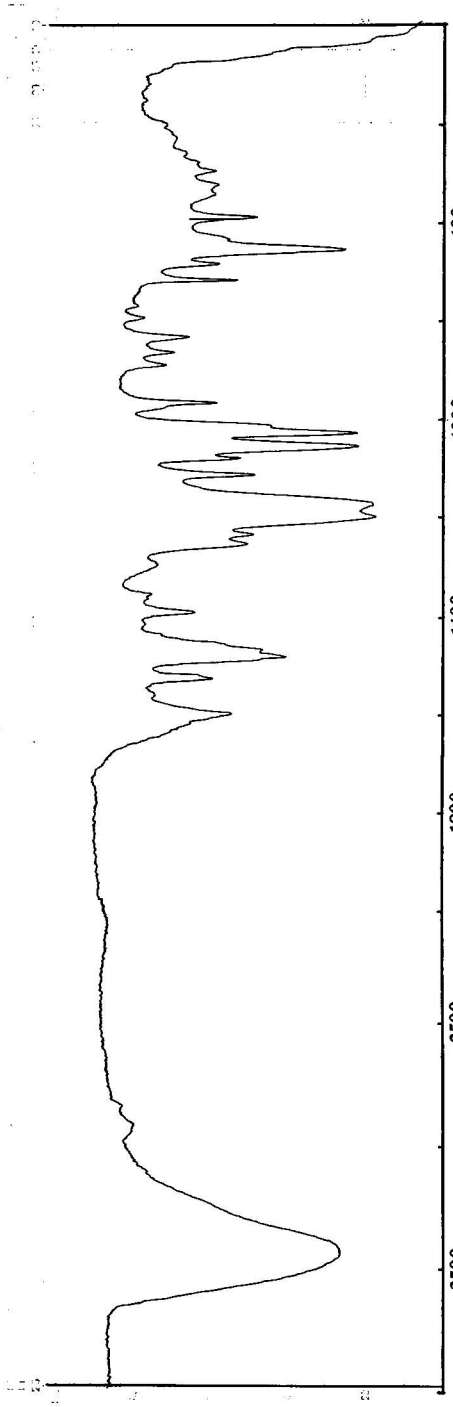


FIG. 25—IR spectrum of FD&C Red No. 40.

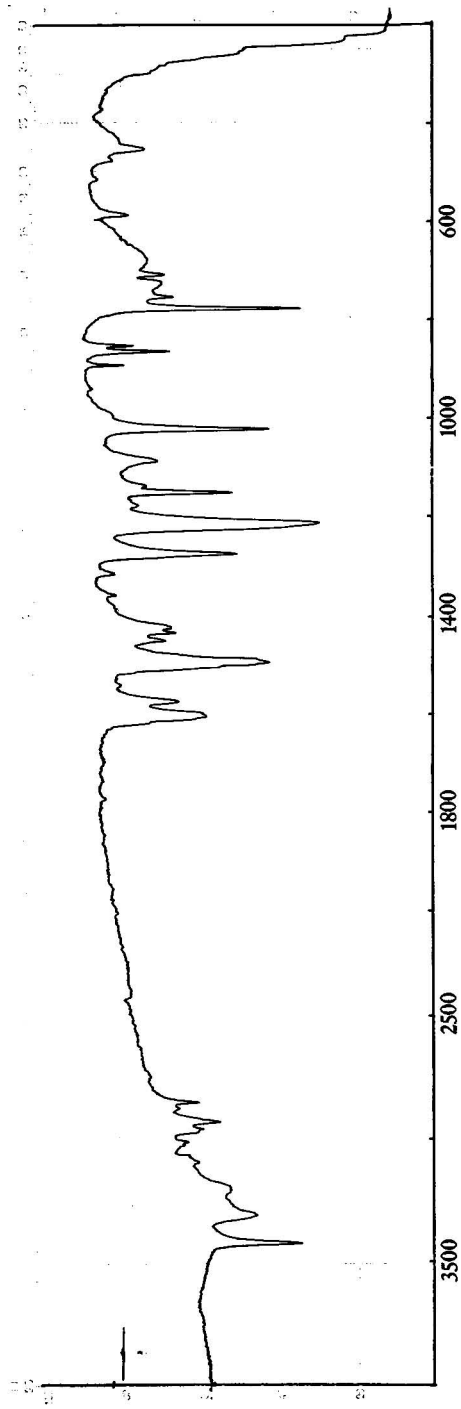


FIG. 26—IR spectrum of cresidine.

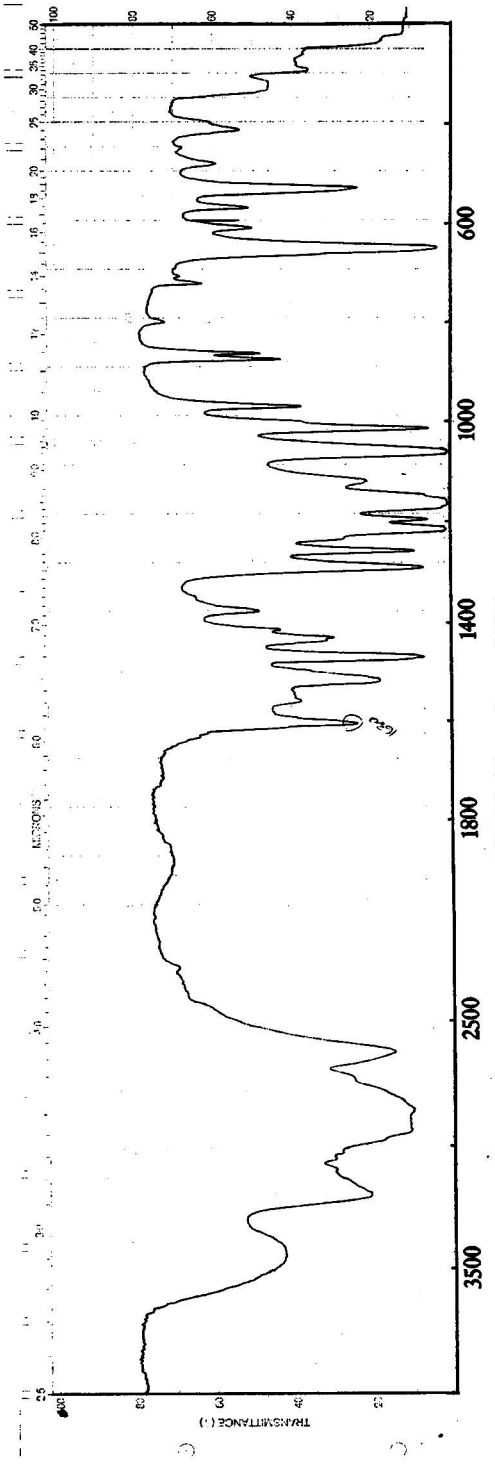


FIG. 27—IR spectrum of CSA.

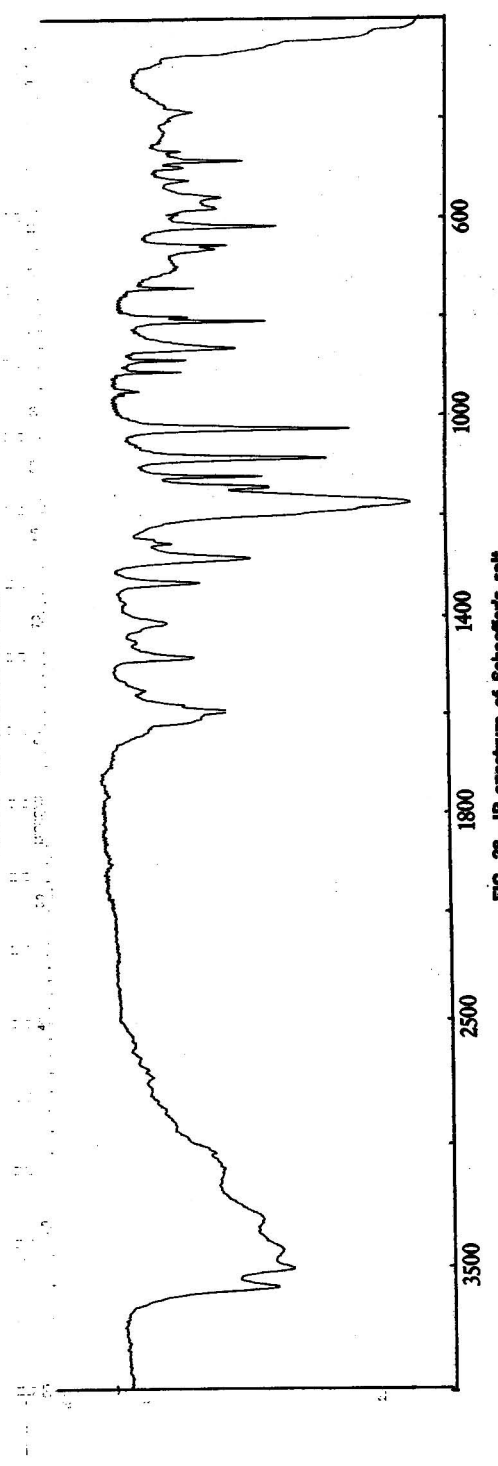


FIG. 28—IR spectrum of Schaeffer's salt.

Table 1. Spectral data for FD&C Red No. 40 and its intermediates and subsidiary dyes

Compound	Acid	Absorptivity (A/g-L)	$\epsilon \times 10^{-4}$ (L/mole-cm)	Neutral	Absorptivity (A/g-L)	$\epsilon \times 10^{-4}$ (L/mole-cm)	Base	Absorptivity (A/g-L)	$\epsilon \times 10^{-4}$ (L/mole-cm)
Visible									
Cresidine + cresidine	520	100	26.9	415	61	16.3	415	64	17.2
Cresidine + 2-naphthol	505	75	21.8	505	76	22.2	452	55	16.1
CSA + 2-naphthol	493	55	21.6	493	60	23.6	502	32	12.6
Cresidine + Schaeffer's salt	497	46	21.4	497	54	21.4	427	23	11.4
Cresidine + G-salt	500	47	23.3	500	48	23.8	500	40	19.8
Cresidine + R-salt	512	48	23.8	515	49	24.3	451	22	10.8
CSA + G-salt	503	43	25.7	503	44	26.3	505	16	9.6
CSA + R-salt	515	44	26.3	515	45	26.8	465	21	12.5
CSA + NTA	508	37	26.0	508	37	26.0	437	16	11.0
OAS + Schaeffer's salt	486	42	20.2	490	44	21.2	436	21	10.1
FD&C Red No. 40	497	51	25.3	497	52	25.8	445	26	12.9
Ultraviolet									
Cresidine + cresidine	262	28	7.5	250	34	9.2	250	37	10.0
Cresidine + 2-naphthol	262	41	11.9	262	41	11.9	272	54	15.8
	310	25	7.3	310	25	7.3	347	25	7.3
CSA + 2-naphthol	264	24	9.5	264	26	10.2	271	20	7.9
	309	20	7.9	309	22	8.7			
Cresidine + Schaeffer's salt	315	28	11.0	315	31	12.2	251	86	34.0
Cresidine + G-salt	249	46	22.8	249	47	23.3	260	25	12.4
	331	25	12.9	331	25	12.4			
Cresidine + R-salt	323	14	7.0	323	15	7.4	241	54	26.8
							289	34	16.7
CSA + G-salt	246	37	22.1	246	37	22.1	244	37	22.1
	327	19	11.3	327	19	11.3	259	37	22.1
CSA + R-salt	245	50	29.6	245	51	30.2	243	44	26.3
	321	15	8.9	321	16	9.6	291	28	16.7
							352	13	7.8
CSA + NTA	332	16	11.2	332	16	11.2	247	36	25.2
							294	22	15.4
OAS + Schaeffer's salt	232	57	27.8	232	72	34.8	249	49	24.0
	263	27	13.0	264	28	13.6	279	34	16.4
	309	17	8.4	308	18	8.7			
FD&C Red No. 40	314	23	11.4	314	26	12.9	248	59	29.2
							280	42	20.8

prevent an increasing volatile content during analysis. The volatile content of each dye was determined as well as the carbon, hydrogen, and nitrogen composition. Sodium content was determined by atomic absorption spectrophotometry (AAS). The potassium content of the G-salt subsidiaries was also determined by AAS since the intermediates used are in the form of potassium salts, giving mixed salt products; the exact proportion of each must be known to calculate pure dye content.

All subsidiaries were screened for extraneous colored bands by thin layer chromatography (TLC). Solutions of each dye prepared were applied to a TLC plate coated with silica gel G; the plates were developed in isoamyl alcohol-1,4-dioxane-acetonitrile-ethyl acetate-water-ammonia (20+20+20+20+20+4), the same method used for the analysis of subsidiary dyes in samples of FD&C Red No. 40 submitted for certification (C. Stein, 1974, Food and Drug Administration, Washington, DC, private communication). HPLC was used to detect the presence of unreacted intermediates as well as many of the subsidiary dyes. Strong anion exchange columns with gradients of sodium borate-sodium percholorate buffer solutions were used to achieve separations (2).

Discussion

The general method followed for the synthesis of most FD&C Red No. 40 subsidiaries was inadequate for the preparation of G-salt subsidiaries, and an alternative method was found. The coupling was attempted at several pH values, each yielding a mixed product. 2-Naphthol, substituted by a sulfonic acid group in the 8-position, as in G-salt, is known to couple slowly, probably due to steric hinderance. The diazotates, either cresidine or CSA, are also slow

couplers, since the alkoxyl group in the *ortho* position decreases the positive charge of the diazonium compound and facilitates its decomposition. Apparently, the coupling to G-salt is slower than the decomposition of the diazo compound, giving poor yields of product. Using aqueous pyridine as the coupling medium gave good yields of the desired product. Venkataraman (3) suggested that pyridine activates the coupling component in addition to stabilizing the diazo group as the diazo-pyridinium radical, which then attacks the coupling site.

Results

Visible (VIS), ultraviolet (UV), and infrared (IR) spectra of each prepared subsidiary are shown in Figs. 1–28. VIS spectra were recorded from 700 to 400 nm and UV spectra from 400 to 210 nm; these spectra were obtained in acid solution (A), 1% concentrated HCl; basic solution (B), 1% NaOH in water; and neutral solution (N), distilled water. Cresidine + cresidine and cresidine + 2-naphthol were dissolved in 95% ethanol. IR spectra are expressed in wave numbers (cm^{-1}). KBr pellets were used to obtain all IR spectra. Absorptivity values as well as extinction coefficients in the VIS and UV range have been calculated and are shown in Table 1. These data will be useful to aid in the identification and quantitation of unknown subsidiary dyes appearing during certification analysis of FD&C Red No. 40.

References

(1) *Code of Federal Regulations* (1973) Part 8, Title 21

(2) Singh, M. (1974) *JAOAC* **57**, 219–220

(3) Venkataraman, K. (1952) *Synthetic Dyes*, Vol. 1, Academic Press, New York, NY, p. 424

Quantitative Determination of 4,4′-(Diazoamino)-dibenzenesulfonic Acid in FD&C Yellow No. 5 by Ion Exchange Chromatography

D. DOUGLAS FRATZ
Division of Color Technology, Food and Drug Administration, Washington, DC 20204

An ion exchange chromatographic procedure separates 4,4′-(diazoamino)-dibenzenesulfonic acid (DAADBSA) from FD&C Yellow No. 5 and DAADBSA is determined spectrophotometrically. Recoveries of DAADBSA added to FD&C Yellow No. 5 at levels of 0.01 to 0.4% ranged from 90 to 130%. DAADBSA was found in commercial samples of FD&C Yellow No. 5 in amounts ranging from 0.006 to 0.022%.

4,4′ - (Diazoamino) - dibenzenesulfonic acid (DAADBSA) has been identified as a component of FD&C Yellow No. 6; DAADBSA is formed when diazotized sulfanilic acid couples with undiazotized sulfanilic acid (1). This can occur if an insufficient amount of acid or sodium nitrite is used. High-pressure liquid chromatographic (HPLC) and column chromatographic methods (1–3) have been described for the determination of DAADBSA in FD&C Yellow No. 6.

Since the preparation of FD&C Yellow No. 5 (tartrazine) also has the diazotization of sulfanilic acid as the initial step, the possibility of the presence of DAADBSA in FD&C Yellow No. 5 samples was investigated. The methods used for the analysis of FD&C Yellow No. 6 are not applicable to FD&C Yellow No. 5 because of the great difference in ionic character of the 2 compounds, due to the different structure of FD&C Yellow No. 5 (*see* Fig. 1). An HPLC method has been developed for the determination of DAADBSA in FD&C Yellow No. 5 (M. Singh, 1975, Food and Drug Administration, Washington, DC 20204). Although this method is rapid and precise, the apparatus required is not readily available to all laboratories and the HPLC techniques may be subject to individual variations. Therefore, the ion exchange chromatographic technique described below was developed for the separation and determination of DAADBSA in FD&C Yellow No. 5.

In the method presented, a strongly basic anion exchange microgranular cellulose packing is used. The buffer and eluant are prepared at pH 12 to assure stability of the DAADBSA, since it was shown to be unstable under acid conditions (1) and in neutral buffer (D. D. Fratz, 1976). The uniformity of the microgranular cellulose, compared to powder cellulose used in column chromatography, causes the DAADBSA to elute in a tight band and therefore very low levels can be detected. Since DAADBSA has been found in much lower amounts in FD&C Yellow No. 5, as compared to FD&C Yellow No. 6, detection of these low levels is essential to the usefulness of the method.

METHOD

Apparatus

(a) *Spectrophotometer.*—Cary Model 118, or equivalent.

(b) *Chromatographic tube.*—22 mm id × 10 cm, glass, with ca 200 ml reservoir top, 22 mm medium fritted disk, Teflon stopcock or tubing with clamp.

Reagents

(a) *Anion exchange resin.*—DE32 (diethylaminoethyl) microgranular cellulose (Whatman Ltd, 9 Bridewell Place, Clifton, NJ 07014).

(b) *Buffer.*—0.01*M* phosphate, pH 12. Dissolve 14.2 g Na_2HPO_4 in 1 L water (0.1*M*). Take 100 ml aliquot, add 3 ml 50% (w/w) NaOH, and dilute to 1 L with water.

(c) *Eluant.*—0.01*M* phosphate buffer, pH 12, and 0.1*M* sodium perchlorate. Dissolve 351.6 g $NaClO_4 \cdot H_2O$ in 500 ml water (5*M*). Mix 20 ml of this solution, 100 ml 0.1*M* Na_2HPO_4, and 3 ml 50% (w/w) NaOH, and dilute to 1 L with water.

(d) *DAADBSA.*—Prepare as described by Bailey and Cox (1). Dissolve 10 mg in 1 L eluant, and determine absorptivity at 407 nm.

Determination

Add 50 mg FD&C Yellow No. 5 to 10 ml volumetric flask, dissolve by adding ca 7 ml buffer and swirling, and dilute to volume with buffer.

Prepare slurry of 5.0 g anion exchange resin and ca 100 ml buffer, and pour into chromatographic tube. Let column form and all liquid enter column. To protect top of packing, carefully place

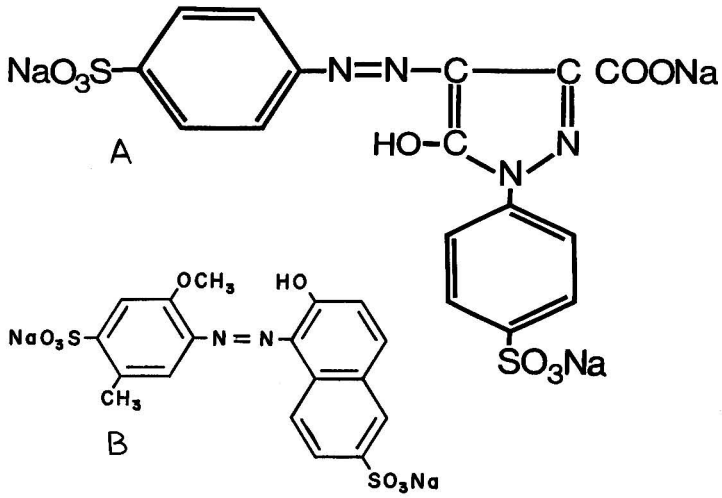


FIG. 1—A, structure of FD&C Yellow No. 5; B, structure of FD&C Yellow No. 6.

22 mm filter paper on column. Wash sides of column with 4–5 ml more buffer, and allow this to enter column.

With column clamped off, add 1 ml (50 mg) sample solution to top of column. Let sample enter column; wash sides of column with ca 10 ml buffer and let wash enter column. Wash sides of tube at least twice again with ca 10 ml eluant, and let wash enter column. When all of dye has entered column, fill reservoir with eluant and allow column to develop. Collect fractions immediately after first eluant enters column.

Discard first 50 ml, which should contain all the FD&C Yellow No. 5. Collect next 15–25 ml, which contains DAADBSA. Measure exact volume of DAADBSA fraction, and determine absorbance at 407 nm, using 2.5 cm or longer cell.

Calculation

$$\% \text{ DAADBSA} = (A \times V \times 100\%)/(W \times L \times A')$$

where A = sample absorbance at 407 nm, V = volume of sample fraction, ml, W = sample weight, mg, L = cell length, cm, and A' = absorptivity of standard at 407 nm A-ml/mg-cm.

Regeneration of Resin

The packed columns may be used for 2–3 determinations. To prepare for reuse, let eluant continue flowing through column until all subsidiary colors have eluted. This process may be shortened by using an eluant containing 0.5*M* sodium perchlorate. Let ca 50 ml buffer elute through column before reuse.

Results and Discussion

Table 1 shows the recoveries of DAADBSA added to a sample of FD&C Yellow No. 5 containing a negligible amount of DAADBSA and analyzed by the anion exchange chromatographic method. Recoveries of DAADBSA added at levels of 0.01–0.40% ranged from 90 to 130%. The FD&C Yellow No. 5 sample with no added DAADBSA showed interference at levels equivalent to 0.002–0.003% DAADBSA.

Table 1. Recovery of DAADBSA added to 50 mg samples of FD&C Yellow No. 5 using ion exchange chromatography

Added, %	Found, %	Rec., %
0.40	0.389	97
	0.395	99
0.20	0.197	99
	0.201	101
0.10	0.102	102
	0.107	107
0.05	0.046	92
	0.053	106
	0.049	98
	0.051	102
0.03	0.029	97
	0.030	100
	0.032	107
	0.032	107
0.01	0.010	100
	0.013	130
	0.009	90
	0.012	120

Three commercial samples of FD&C Yellow No. 5 were analyzed for DAADBSA by HPLC (M. Singh, 1975) and by ion exchange chromatography with the following results (%): 0.030, <0.02, and <0.02 (HPLC) and 0.020, 0.022; 0.009, 0.009; and 0.006, 0.007 (ion exchange, duplicate results). The results are in good agreement, considering the very small amounts of DAADBSA found in the commercial samples.

After the DAADBSA has been eluted from the column, at least 9 different subsidiary colors may separate. None of these interferes with the DAADBSA band. The interference seen in the analysis of the FD&C Yellow No. 5 sample with no DAADBSA added had no visible absorbance maximum, but rather an absorbance which increased slowly from about 425 to 350 nm. Evidence of this interference in a sample containing DAADBSA can be seen as a lowering of the maximum absorbance a few nanometers below the usual 407 nm. The interference, however, is not enough to be detrimental to the determination.

Received May 25, 1976.

This paper was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Acknowledgments

The author thanks C. Bailey for the HPLC results of the commercial samples and J. E. Bailey for the DAADBSA standard.

REFERENCES

(1) Bailey, J. E., & Cox, E. A. (1975) *JAOAC* **58**, 609–613
(2) Fratz, D. D., & Bailey, J. E. (1976) *JAOAC* **59**, 12–13
(3) Marmion, D. M. (1975) *JAOAC* **58**, 719–724

REVIEW OF AFLATOXIN METHODOLOGY

A Review of Sampling Plans and Collaboratively Studied Methods of Analysis for Aflatoxins

PIETER L. SCHULLER, WILLIAM HORWITZ,* and LEONARD STOLOFF*
Laboratory for Chemical Analysis of Foodstuffs, National Institute of Public Health, PO Box 1, Bilthoven, The Netherlands
** Bureau of Foods, Food and Drug Administration, Washington, DC 20204*

Aflatoxins are the only food contaminants being monitored routinely on an international scale with methods operating at the order of magnitude of 10 μg/kg. At this concentration level, methods of analysis which can achieve coefficients of variation of 30–40% with recoveries of 70% or greater in interlaboratory collaborative studies can be considered eligible for referee status. In most cases, sample reproducibility is the variable limiting the reliability of methods of analysis. The inherent uncertainty of the identity of chromatographically separated entities requires the application of confirmatory tests to verify that the characteristic measured results from the presence of aflatoxin. The methods are also inoperable without a verification of the identity, purity, and concentration of the reference standards used. Screening methods which reliably eliminate negative samples from further consideration are indispensible for the practical operation of monitoring programs.

The purpose of this paper is to review the availability of sampling plans and collaboratively studied methods of analysis for aflatoxins in foods and feeds. It is a condensation and updated version of a longer document (1) intended to supply background information for recommendations of acceptable *Referee Methods* for the prevention and settlement of disputes in international trade. The original document was prepared for the Food and Agriculture Organization (FAO)/World Health Organization (WHO) ad hoc Expert Consultation on Methods of Sampling and Analysis, held in Rome, Italy, Jan. 12–16, 1976. This consultation, in turn, was held in response to a recommendation of the United Nations Conference on the Environment held in Stockholm in 1972 to strengthen the work of the Codex Alimentarius Commission (CAC) and to improve the capabilities of FAO and WHO to assist developing countries in food control.

Aflatoxins are a group of toxic compounds produced by the mold *Aspergillus flavus*, from which the name was derived, and the closely related *A. parasiticus*. Early studies of the toxic agent in peanut meal demonstrated that the toxic factor could be separated chromatographically into 4 distinct spots (2). All 4 components were given the name "aflatoxin" relating to their common origin, with the addition of the letters "B" (blue) and "G" (green) to refer to their fluorescent color and the subscripts "1" and "2" to refer to their relative chromatographic mobility. To date, at least 17 closely related compounds have been isolated, characterized, or synthesized. Practically all of them contain the difuranocoumarin moiety with the lactone ring oxygen conjugated with a double bond. Since only aflatoxins B_1, B_2, G_1, G_2, M_1, and M_2 have been found as contaminants in foods and feeds, collaborative studies of methods of analysis have been confined to these compounds. For confirmation of the identity of aflatoxin B_1, the formation of the hemiacetal aflatoxin B_{2a} has been found useful. The structures of these compounds are given in Fig. 1.

Two types of tests have been developed for the detection and determination of aflatoxins: biological and chemical. The bioassay techniques are only semiquantitative and generally nonspecific. Those currently available are unsuitable for routine screening purposes and do not respond to the low levels required for food control purposes. The chemical assay techniques, although more reliable and faster, can produce false identifications, particularly on commodities with which the analyst has had little experience. Chromatographically isolated spots, tentatively identified as aflatoxins, can be verified as to

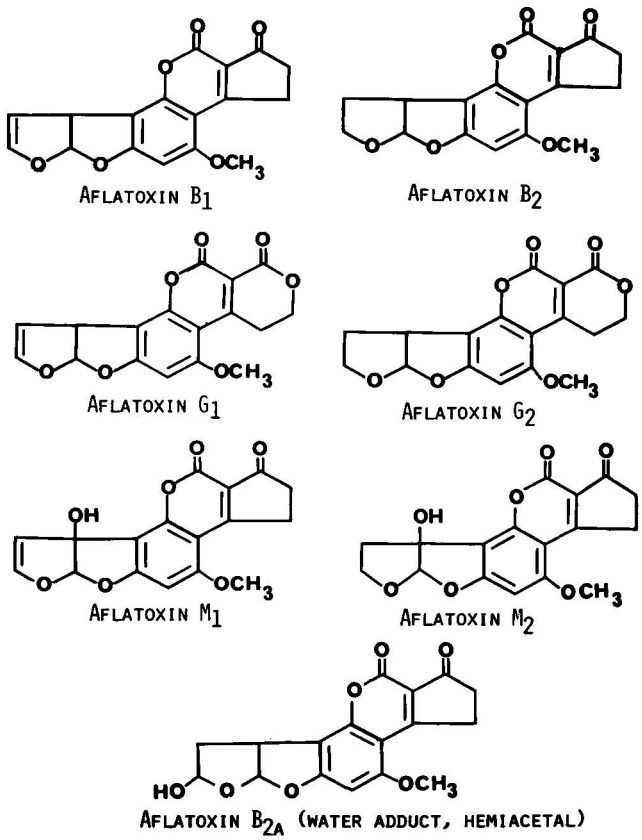


FIG. 1—Structures of aflatoxins of analytical interest in food and feed contamination.

identity with progressive certainty by additional chromatography with different solvent systems and by co-migration with internal standards, by derivative formation, and finally by mass spectrometry. The toxicity of the compound can be verified by bioassay. The most perplexing problem in the analysis for aflatoxin in contaminated materials is that of sampling.

The natural occurrence of aflatoxins in foods and feeds must be distinguished from its laboratory production in various commodities. The literature in this respect is confusing and must be interpreted with care. The *A. flavus* group of molds is ubiquitous and members have been isolated from a wide variety of sources. Many of these isolated molds have been described as capable of producing aflatoxin, but often the source from which they have been isolated was not analyzed for the prior presence of the toxin in the substrate. Moldiness is not always accompanied by the production of toxin.

Sampling

Sampling is undoubtedly the most important contributor to the variability of analyses for aflatoxin in agricultural commodities. This fact arises from the inherent discontinuous nature of the distribution of aflatoxin in raw agricultural commodities.

Sampling from a Lot

The difficulty of sampling particulate commodities for aflatoxin derives from the normally small proportion of contaminated kernels in a lot, as suggested by Robertson *et al.* (3), and the considerable variation in the amount of

aflatoxin among infected units. This variation has now been demonstrated for peanuts (4), cottonseed (5), and corn (6). Levels of the order of magnitude of 1,000,000 μg/kg in a few individual kernels were reported in these investigations.

The problem of sampling shelled peanuts for aflatoxin has been investigated extensively, both theoretically and experimentally, in a remarkable series of papers by Whitaker and his colleagues. In the first paper, Whitaker and Wiser (7) point out that the distribution of aflatoxin in shelled peanuts resembles the characteristics of the incidence of contagious diseases, which have been studied successfully using the negative binomial distribution. The negative binomial probability function describes a situation with high probabilities of zero counts and low probabilities of very high counts. The general applicability of this distribution was tested with data from 20,000 lots of shelled peanuts analyzed by the U.S. Department of Agriculture (USDA) in 1967 (8) and from 10,767 lots analyzed in 1969 (9). With this information, efficient sampling procedures (sample size and number of samples per lot) can be devised to determine within acceptable confidence limits if the true average level of aflatoxin in a lot is above or below a given tolerance level. The fit of the negative binomial distribution to actual sample analyses was tested (10) by subdividing each of 29 lots of shelled peanuts into ten 12 pound (lb) (5.4 kg) samples and determining the aflatoxin content of each of the 290 samples. The observed variability indicated that the assumed distribution is a reasonable model for the actual distribution.

Whitaker *et al.* (9) summarized the procedure for developing sampling plans and evaluated the 4 sampling plans (2 single and 2 sequential) used in the United States to control aflatoxin content in shelled peanuts during the period 1969–1972. The sample size varied from 10 lb (4.5 kg) to 48 lb (22 kg). The current (1975) plan (11) is a 3-step sequential procedure (three 48 lb samples).

The assumptions on which these plans are based are:

(*1*) The reliability of the determinations of the aflatoxin distribution in commercial lots of shelled peanuts from the 1969 crop, as measured from the analyses of ten 12 lb samples (each containing about 10,600 kernels) from each of 29 lots;

(*2*) The applicability of the negative binomial distribution model and the accompanying assigned constants, e.g., 99.9% of the kernels contain no aflatoxin and the remaining 0.1% are contaminated at an average level of 50 μg/kg;

(*3*) All samples are drawn in a random manner;

(*4*) All of the errors are due to sampling; none are ascribable to subsampling or to analysis.

The data base for the calculations (8, 9) was from years with an unusually high incidence of aflatoxin in peanuts. Therefore, the use of this data base in developing the sampling plans affords a high safety factor. The correspondence of the experimental data to those calculated from the model equation appears to be satisfactory (10), particularly when it is considered that the method of analysis has a total overall relative error of about 30% and a within-laboratory relative error of 15–20%. With such a large analytical and subsampling error, many skewed distributions should also fit the experimental data fairly well. All instructions for sampling emphasize the need for obtaining a random sample (which is not the same as a haphazard sample). An experimental study of the relationships of sampling, subsampling, and analytical variances as a function of the size of sample and subsample and number of analyses has also been described by Whitaker *et al.* (12). For the test procedures used in this study, sampling constitutes by far the largest single source of error, followed by subsampling, and then by analysis. The proportion of error due to sampling becomes greater as the aflatoxin concentration becomes lower. Therefore, the model of Whitaker and Wiser (7) appears to be a valuable tool for selecting and comparing sampling plans.

Tiemstra (13) studied the variability within 550 lots of the 1967 crop of peanuts sampled in triplicate, using 10 lb samples. He, too, concluded that lot sampling was the critical variable in determining the aflatoxin content of a lot. Tiemstra found that a log normal distribution (less peaked than the negative binomial but still very skewed) represented his data very well.

Velasco *et al.* (14) provided the basic variability data needed for developing sampling plans for cottonseed and the relationships of the com-

ponent variances for this commodity were studied by Whitaker *et al.* (15).

The procedures recommended by the International Association of Seed Crushers (16) for sampling oilseeds, recognized as good practice for obtaining samples to estimate most of the factors affecting oilseed quality, are not adequate for aflatoxin control. Representative sampling of milled commodities is not as difficult as with the original seed. With oilseed cakes, for example, some breaking and mixing of kernels occur during processing. Cereal grains present intermediate sampling difficulties because of smaller kernel size and, in the case of corn, because of a higher incidence of contaminated kernels (6) than peanuts. Well mixed processed products, such as peanut butter, present no sampling problem; neither do milk and milk products, because of their original fluid nature.

The sampling plans which have been developed on statistical grounds are based upon the distribution of aflatoxin in peanuts as found in the United States during certain crop years and using the limit established by the U.S. Food and Drug Administration (FDA). The legal, geographic, and practical requirements of other countries and the characteristics of specific commodities will influence the selection of other appropriate sampling plans.

Sampling plans developed for regulatory purposes have little relationship to sampling plans for dietary or surveillance purposes. For dietary surveys, there are no critical levels, no definition of acceptable lot, and no consumer and producer risks. Averaging of large numbers of samples in a survey automatically reduces all errors by the square root of the number of samples. Survey samples suffer from other types of errors, e.g., the level of aflatoxin contamination in "cookpot" samples is considerably lower after dilution with nonsusceptible foods than with lots of bulk commodities. In fact, as pointed out by Barnes (17), there is little point in analyzing food eaten at any particular time to determine if it contains aflatoxin, since chance plays a great part in determining the degree of contamination of the portion consumed. For public health purposes, information should be obtained at a much earlier stage, with adequate surveys of food at harvest and storage, so that the populations most heavily exposed regularly or intermittently to food contaminated with mycotoxins may be identified.

Conclusion.—Development and application of sampling plans require a promulgation by governmental or Codex authorities, or an agreement between parties regarding the following factors: a critical level (control, tolerance, guideline, etc.) for aflatoxin(s); a definition of a good (acceptable) and a bad (rejectable) lot; and a statement of the acceptable or desired consumer and producer risks. In the absence of this information, selection of any sampling plan will be arbitrary. The evaluation of Whitaker *et al.* (9), which includes a comparison of operational and financial aspects, and the experience gained in the United States with the use of several plans for the control of aflatoxin contamination, if supplemented by operational and financial information on more recent sampling plans, will provide a rational basis for the selection of a sampling plan.

Laboratory Scale Sampling (Subsampling) and Preparation of Sample for Analysis

In accordance with Whitaker's sampling plans, large samples should be taken, for regulatory purposes, to reduce sampling error. Fifty and 60 lb (23 and 27 kg) portions are taken for brazil and pistachio nuts in the United States. The current U.S. peanut program (11) uses a sequential sampling plan which requires that three 48 lb (22 kg) samples be submitted to the laboratory. Only the first is ground until the result of the analysis is known. For survey purposes, 1–5 kg samples are usually taken. In all cases, it is important that the entire sample supplied to the laboratory be ground and mixed. The particle size of the first reduction is not critical, but the amount of subsample taken for further size reduction will depend upon that particle size. The ultimate subsample from which the analytical sample is taken must be fine enough to pass through a U.S. Standard No. 20 sieve (ISO 850 μm).

Stoloff *et al.* (18, 19) compared the effectiveness of various comminuting devices for nuts by using nuts made radioactive by neutron activation to simulate a desired incidence of contamination. The distribution of radioactivity in selected portions after grinding and mixing provided a precise means of measuring within-

sample variability. A number of techniques have been described for the preparation of samples for analysis (20, 21). The equipment now most commonly used for peanuts in the United States is the Dickens-Satterwhite subsampling mill (22) and for in-shell nuts, the Hobart vertical cutter-mixer. For some products such as grains, an ordinary disk mill is satisfactory for size reduction, followed by mixing. Other effective techniques are freezing the sample in liquid nitrogen, using grinding aids such as oyster shell, grinding tree nuts in-shell, grinding with a small amount of solvent (19, 23), and preliminary defatting for high-fat products such as copra.

Sample sizes used for the actual analyses have ranged from 20 to 100 g in various methods. The compromise between solvent economy and a representative sample has been made at 50 g (3).

Biological Tests

Since its initial discovery and isolation, aflatoxin B_1 has been evaluated in a greater variety of biological systems than any other mycotoxin. Almost 1000 papers have appeared describing the effects of aflatoxins in systems ranging from subcellular enzyme systems through cell cultures and microorganisms to almost every laboratory animal.

In the original biological test (24), newly hatched ducklings were used as the test animal for determining the presence of aflatoxin isolated from suspect food, with bile duct hyperplasia as the specific, measured response. Wogan (25) has shown that this test is, at best, only semiquantitative. The lowest dose level of 0.4 μg/day administered for 5 days represents a minimum intake required to induce a detectable bile duct lesion and is the limit of detectability by this technique in the duckling. The test is also effective for detecting aflatoxin M_1 in fluid milk and powdered milk (26) after toxin isolation. Limited information is available on the response of this test (and other biological tests) to other aflatoxin-related compounds.

Burmeister and Hesseltine (27) surveyed over 300 species of microorganisms for their sensitivity to aflatoxin. One strain of *Bacillus brevis* and 2 of *Bacillus megaterium* were most sensitive to aflatoxin. This observation was translated into a zone of inhibition assay method (28) which was not sufficiently reproducible in collaborative studies to warrant further investigation.

Of all the biological tests, the chicken embryo bioassay has proved to be the most useful. The assay is reproducible and typical lesions are observed in the embryo with subacute levels of aflatoxin B_1, less than 0.1 μg/egg. The assay has been used routinely by the U.S. FDA to confirm the toxicity of aflatoxin B_1 isolates from contaminated samples. The correlation with the chemical confirmation test has been 100% with over 500 samples. A minimum of 2 μg, and preferably 5 μg, of isolated aflatoxin B_1 is required for an adequate number of eggs at suitable levels to establish toxicity.

Conclusion.—The chicken embryo bioassay has been studied collaboratively (29). In general, a linear dose-response relationship was exhibited and good agreement was obtained with the LD_{50}, slope, and associated standard errors. It is interesting to note that the LD_{50} for pure aflatoxin B_1 was 0.02 μg/egg and that for aflatoxin B_1 isolated from peanut butter was 0.04. The method has been adopted as official final action by the Association of Official Analytical Chemists (AOAC) (21, **26.073–26.078**) and can be recommended as a referee method for confirmation of toxicity of isolated aflatoxin B_1 preparations.

Chemical Assay

General

The aflatoxins are intensely fluorescent when exposed to longwave ultraviolet (UV) light. This fortuitous circumstance permits the detection of these compounds at extremely low levels (ca 0.5 ng/TLC spot) and provides the basis for practically all the physicochemical methods for their detection and determination. Most methods use the comparison of intensity of fluorescence of a standard with that of the sample for estimation of the amount of toxin present. Comparison of sample and standard spots is always made on the same thin layer chromatographic (TLC) plate, since the adsorbent layer affects the fluorescent intensity (30). The "dilution to extinction" technique (31) used in the first published methods is an inherently less accurate procedure, which is gradually being phased out of use. Visual comparison of afla-

toxin spots on TLC plates under ideal conditions only permits distinguishing intensity differences in spots differing in amount by more than 20% (32). This is the limit of normal visual acuity. In the same study (32) the coefficient of variation of visual estimation of the fluorescent intensity of spiked extract spots on TLC plates was found to be 28%. Both accuracy and precision of aflatoxin analysis can be enhanced when instrumental measurement of the intensity of the fluorescence is made. A number of publications describe different types of fluorodensitometers (30, 33–37). Measurements of replicate standard spots made with these instruments, set for ultimate possible precision, gave a pooled coefficient of variation from 4 separate studies (34–37) of about 6%; measurements of spiked peanut butter extracts (30, 33, 37) gave a pooled coefficient of variation of about 9%; and a single study of replicate analyses of cottonseed (32) gave a coefficient of variation of 13% for the densitometric measurements. However, most collaborative studies show about the same overall coefficient of variation, about 30%, for both visual and densitometric measurements, indicating that other errors predominate. Method compilations, therefore, permit the use of either way of reading the aflatoxin spots.

There have been attempts to recover aflatoxin by preparatory TLC for measurement by polarography, fluorometry, spectrofluorometry, or spectrophotometry, but these techniques have been practical only for such high concentrations as are found in cultures. There is some deterioration of aflatoxin from photooxidation when exposed to UV light for visualization (38).

In the United States, Canada, Federal Republic of Germany (food only), and Sweden, aflatoxin is reported as total aflatoxins ($B_1+B_2+G_1+G_2$); most other countries report only aflatoxin B_1. The use of total aflatoxins was based upon the initial prudence required when knowledge of the relative risk from the several compounds was lacking. Although aflatoxin B_1 is the most toxic aflatoxin, it is not the most fluorescent. A ratio of the solid state fluorescence, as reported by Pons *et al.* (30), for $B_2:G_2:B_1:G_1$ is 4.8:2.7:2.0:1.0, although the ratios may vary considerably with the nature of the developing solvent (37) and the gel used for TLC (30). These ratios are considerably different in solution, for example, the corresponding values in the same order in aqueous solution are 8:13:1:5 (24). The precision of the determination of total aflatoxin concentration is also somewhat better than that for the individual compounds (30, 36), probably from compensation of errors. For accurate measurements, each spot must be compared by interpolation with a graded series of spots of the appropriate reference standard (36).

All the analytical methods for mycotoxins contain the following basic steps: sample preparation, extraction, lipid removal, cleanup, separation, and quantitation. Since there is considerable overlap in the various methods, most of the published reviews (20, 39, 40) examine the different procedures by basic steps. Because of the nature of some commodities, methods can sometimes be simplified by omission of unnecessary steps; other commodities, because of the presence of specific interferences, e.g., gossypol in cottonseed and theobromine in cacao, may require additional steps in the method. Frequently, because of differences in laboratory environment, introduction of new batches of reagents (e.g., silica gel, chloroform), and variations between commodities, it is necessary to adjust the composition or proportions of the TLC developing solvents to obtain adequate separation of the aflatoxins or aflatoxin derivative spots from extract interferences as discussed in the introduction to the AOAC Natural Poisons chapter (21), and specifically in sec. **26.010** of that chapter.

The analytical methods may be complicated by several factors: (a) the distribution of aflatoxin in natural products is usually heterogeneous, creating an unusual sampling problem; (b) aflatoxins are found in agricultural commodities which may be sufficiently different to require different extraction procedures; (c) the amount of toxin present is usually in the μg/kg range, requiring elimination of a large mass of "inert" material; (d) extracted aflatoxin is usually accompanied by pigment and fluorescent interferences, necessitating a suitable cleanup step; and (e) depending upon the history of the sample and the cleanup steps, the observations may be nonspecific and require confirmation of identity of the fluorescent spots.

Numerous methods of analysis have been reported for the determination of aflatoxins in human and animal food. Many are minor modifications of basic methods, adapted to local

commodities or problems. The methods discussed below were chosen on the basis that referee methods preferably should have been subjected to interlaboratory collaborative studies (41). A collaborative study measures the ability of normally skilled analysts in different laboratories to reproduce each other's results, utilizing the same method on portions of the identical material. The interpretation of the results and the judgment of the suitability of the tested methods is an administrative decision by governments and those agencies which utilize the studies. For legal purposes, the results of the studies must be published or otherwise made available for review and reinterpretation by those affected by the use of those methods of analysis.

As a result of extensive surveillance work by the U.S. FDA, Fischbach and Rodricks (42) reported that the potential for finding aflatoxin contamination is established for the following common commodities: peanuts, cottonseed, copra, corn, and various tree nuts (pistachio, almond, walnut, pecan, filbert, and brazil). When aflatoxin-contaminated ingredients are used in dairy feed, aflatoxin B_1 is metabolized in part to aflatoxin M_1, some of which is excreted in the milk (43–45). If the contaminated ration is fed to laying hens, a part of the aflatoxin B_1 is transmitted to the egg (46). When given to meat animals, a small fraction of aflatoxin B_1 and its metabolic M_1 is found in the edible tissue, particularly the liver (47, 48).

Methods for vegetable products, which are subject to direct contamination, are usually designed to operate at levels of aflatoxin greater than 1 μg/kg; methods for animal products, which contain residues and metabolites of ingested aflatoxins, are usually designed to operate at levels 1 or 2 orders of magnitude lower.

Three types of methods are required for analyzing foods for the presence of mycotoxins:

(*1*) *Screening Methods.*—Methods which will rapidly and reliably eliminate ("screen") a large number of negative samples to permit the application of more time-consuming quantitative methods to the relatively few samples which give a positive response.

(*2*) *Presumptive Methods.*—Methods which provide qualitative and quantitative results by relatively nonspecific procedures. The qualitative results may become very reliable through history of application to a specific commodity, laboratory experience, and application of specific isolation and cleanup techniques.

(*3*) *Confirmatory Methods.*—Methods which provide positive identification or verification of the identity of the material under investigation.

Screening tests are not ordinarily eligible for referee status since they are designed to provide only a positive or negative response.

The "CB," "BF," and "cottonseed" methods are the 3 most generally applied presumptive tests for aflatoxins. The CB method (21, **26.014–26.019**), originally designed for peanuts, provides a relatively clean isolate. It simultaneously extracts the sample with water and extracts the water with chloroform, transfers the lipids and aflatoxins to a silica gel column, selectively elutes the fat with hexane and the pigments and other interfering materials with absolute ether, and finally strips the aflatoxins from the column with 3% methanol in chloroform. The BF method (21, **26.020–26.024**), also originally designed for peanuts, is faster and more economical of solvents but provides a poorer cleanup. It simultaneously extracts and defats the sample with a 2-phase system, aqueous methanol-hexane, and then partitions the aflatoxins from the aqueous phase into chloroform, leaving lipids and pigments in the hexane and aqueous methanol. After concentration of the aflatoxins in the chloroform by evaporation, both methods separate the aflatoxins by TLC and quantitate them by fluorometry on a TLC plate by visual or densitometric comparison with standards. The cottonseed method (21, **26.048–26.056**; **26.A09–26.A16**) contains features of both the CB and BF methods in that it employs an aqueous polar solvent (acetone-water) as does the BF method, and a silica gel chromatographic column as does the CB method, and in addition uses a lead acetate precipitation in the initial extract to remove specific interferences such as gossypol. This method appears to be the most precise of those widely used. A flow diagram of these 3 widely used methods is shown in Fig. 2.

In The Netherlands, the method of choice (49) uses a 2-dimensional TLC approach (37) to achieve the desired separation of aflatoxin B_1 from interferences. This technique provides considerable saving of time over the use of column chromatography for extract cleanup; on the other hand, a separate TLC plate is required for each sample.

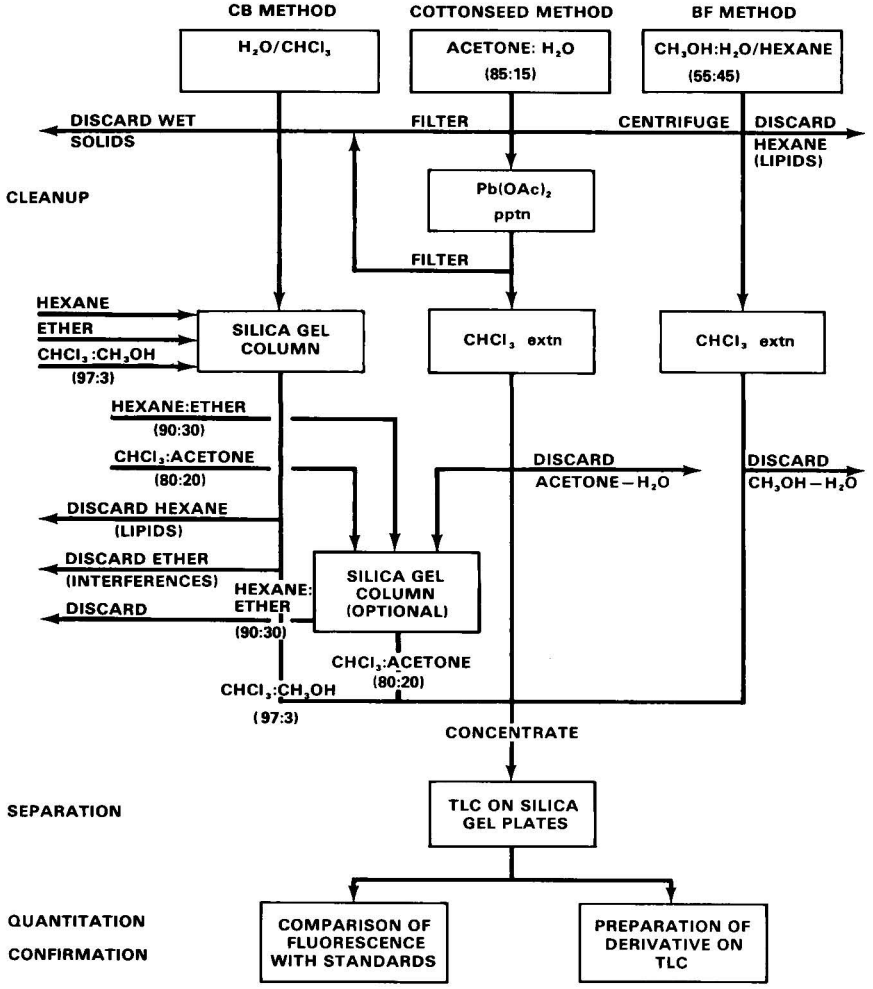


FIG. 2—Flow diagram of the operations of the CB, cottonseed, and BF methods for the analysis of aflatoxins.

The Attributes of Referee Methods

Referee methods are those judged suitable for use by governments in regulatory control and for use in settling or avoiding disputes. They are recommended for inclusion in agreements to abide by the results of analysis by the referee method in combination with a suitable sampling and decision plan.

The operational characteristics of the methods are determined from an interlaboratory collaborative study, involving at least 6 laboratories and at least 5 samples, which is designed to obtain the following types of information:

(*a*) *Accuracy*, through the calculation of the mean recovery of known amounts of added aflatoxins which have been carried through the entire method (spiked samples). This test applies only when "pure" compounds are added. Use of a naturally contaminated sample whose aflatoxin content has been determined by the method under test does not provide an independent determination of recovery.

(*b*) *Precision*, through the calculation of the between-laboratory coefficient of variation and preferably the within-laboratory coefficient of variation by the inclusion of analyst replications

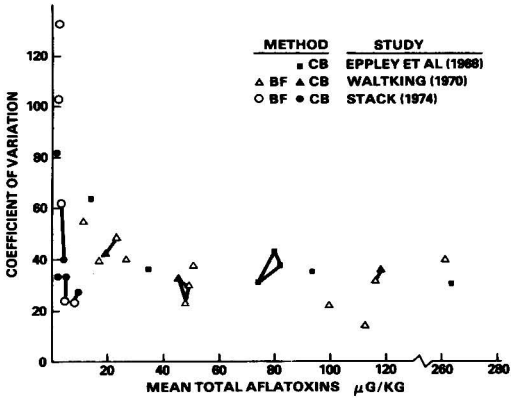


FIG. 3—Overall coefficient of variation as a function of mean total aflatoxins level in peanuts and peanut products, as determined by collaborative studies of the CB and BF methods. Interconnected points indicate replicate samples.

beginning with separate analytical portions (repeatability). These terms can also be obtained by the submission of blind replicates (identical samples submitted as separate unknowns), a preferable procedure.

(*c*) *False negatives*, through the inclusion of samples with aflatoxins concentrations near the assumed limit of detection. An acceptable limit of detection should be chosen so that at that level not more than 1 incorrect decision per group of 10 decisions, or fraction thereof, should be tolerated for consideration as a referee method. Referee methods are not operable near the limit of detection. They should only be used in an area of acceptable precision where "no" false negatives or false positives are observed.

(*d*) *False positives*, through the inclusion of uncontaminated samples. Such samples also provide information on the inherent variability of the *commodity* blank, as produced by interfering materials inherent in the commodity. A criterion similar to (*c*) should be used for the acceptable limit of detection. Sometimes it is impossible to distinguish commodity background from an inherent low level of contamination. The limit of detection is determined by either the false negative or false positive criterion, since there is only a single limit of detection.

(*e*) *Specificity*, by tests for confirmation of identity. A criterion as in section (*c*) should be used for the acceptable limit of applicability.

The values assigned to these criteria of suitability are a function of the current state of the art. It appears that a method which demonstrates a recovery of at least 70% and a coefficient of variation no greater than 40% at a total aflatoxins level of at least 10 μg/kg (*see* Figs. 3–8) represents the current state of methodology. Methods meeting these criteria are acceptable as referee methods. However, this is not considered herein as an absolute rule but as a rough guide for approximation of suitability. Methods not meeting these criteria may also be

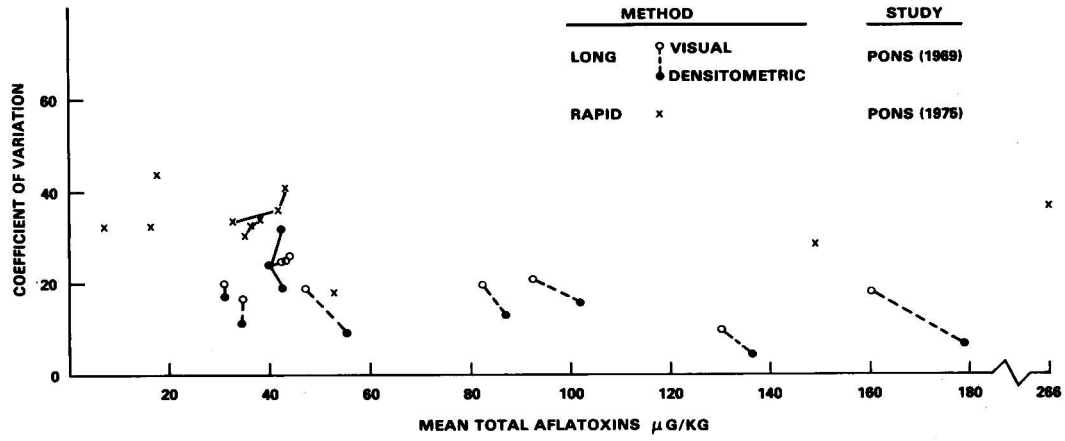


FIG. 4—Overall coefficient of variation as a function of mean total aflatoxins level in cottonseed and cottonseed products as determined by collaborative studies of the "original" and "rapid" cottonseed methods. Points interconnected by solid lines indicate replicate samples.

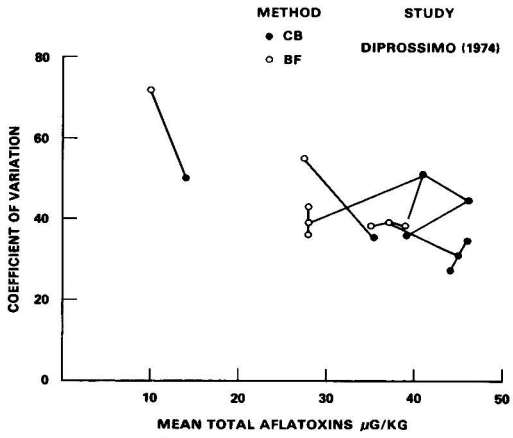


FIG. 5—Overall coefficient of variation as a function of mean total aflatoxins level in pistachio nuts as determined by a collaborative study of the CB and BF methods. Interconnected points indicate replicate samples.

acceptable when there is no alternative. For consideration of a method in such cases, the major conditions and qualifications are as follows:

For a commodity subject to a high incidence of aflatoxin contamination, the need for practical control procedures may require the acceptance of less strict criteria values; with a commodity that rarely requires surveillance, deficiencies in accuracy may be remedied by using a correction factor, and deficiencies in reproducibility may be alleviated through the analysis of more samples rather than by investing resources in improving the method.

At concentration levels approaching the limit of detection, lower performance standards often must be accepted as, for example, in the case of milk products; at higher concentration levels, better reproducibility, though not necessarily better accuracy, is expected.

The coefficient of variation considered is the "total error," which includes the between- and within-laboratory components as well as any interactions. Most of the total error in aflatoxin analysis is attributable to within-laboratory components. The within-laboratory (operator) error may be reduced considerably (down to the limitation imposed by sampling variability and commodity background) by numerous techniques, such as increasing the number and size of samples, further reduction of particle size, more effective mixing, larger analytical samples, replications, averaging results, and compositing

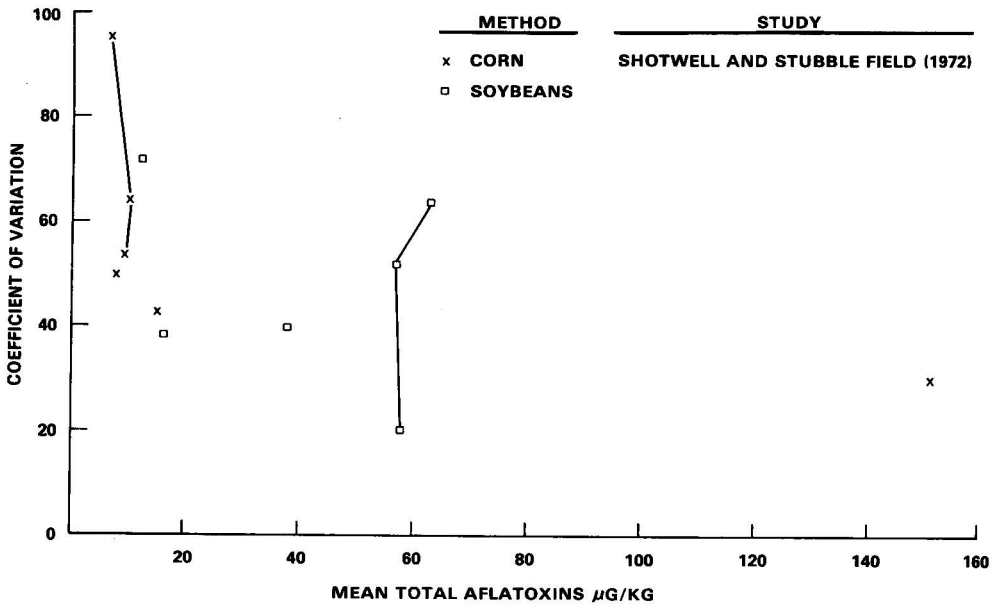


FIG. 6—Overall coefficient of variation as a function of mean total aflatoxins level in corn and soybeans as determined by collaborative study. Interconnected points indicate replicate samples.

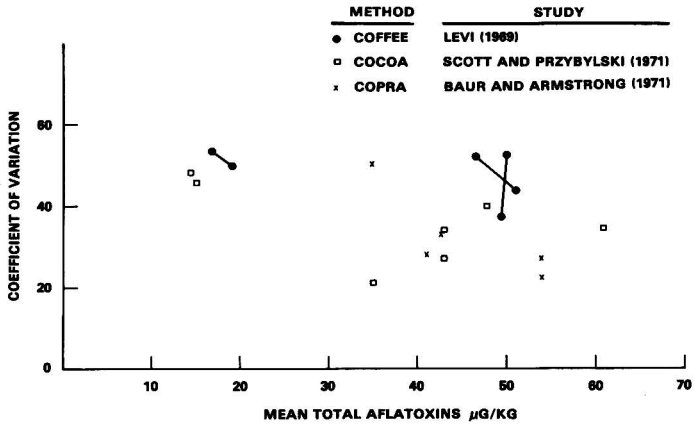


FIG. 7—Overall coefficient of variation as a function of mean total aflatoxins level in coffee, cocoa, and copra products as determined by collaborative studies. Interconnected points indicate replicate samples.

samples. The major between-laboratory error is likely caused by differences in reference standards. To reduce between-laboratory error, particular attention must be paid to the initial accuracy and continuing integrity of the standards, as detailed in **26.009** and **26.012** (21).

However, it should be kept in mind that replications near the end of a method (e.g., duplicating the TLC from the same cleaned up extract) are not nearly so effective in improving reliability of the value of the *lot* as is analyzing 2 separate samples from that lot. This is true despite the fact that the duplicate results from the same extract are most likely to be closer together than the results from 2 separate samples from the same lot.

In the analysis of very small quantities, the chance of error (loss of analyte or extraneous contamination) is so great that the analyst must constantly exercise quality control over his operations by using standard samples to accompany his unknowns, carrying blanks, standards, and spiked samples through all operations simultaneously, and participating in check sample series to demonstrate or verify his proficiency. A reasonable fraction of the analytical time must be invested in continuous quality control of laboratory operations.

The specific values for the various attributes derived from an interlaboratory study represent the best values from a group of presumably representative laboratories. The capability of an individual laboratory or analyst to attain such performance is not automatic. Any given analyst's performance may be better or worse. Laboratory and analyst performance must be demonstrated by a conscientious effort, which may be a special task or part of the quality control program. Performance can often be improved by practice and experience, but only to a minimum level determined by operations such as sampling and manipulations or by instrumental (or visual) limitations. Performance better than this minimum may be suspected as arising from unreported averaging or compositing, or from the "quantum jumps" imposed by

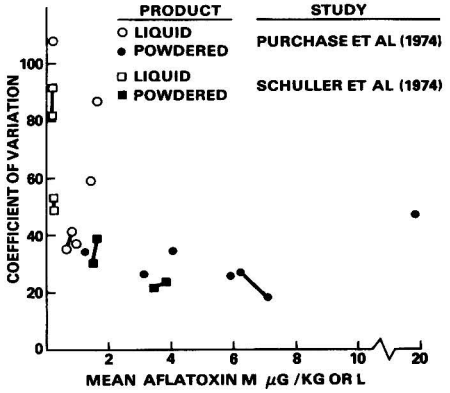


FIG. 8—Overall coefficient of variation as a function of mean total aflatoxins level in liquid and powdered milks as determined by collaborative studies. Interconnected points indicate replicate samples.

the succession of the discrete values of the standard spots.

Finally, sampling, which is often not the responsibility of the laboratory, rather than laboratory operations often contributes the greatest proportion of the variability in aflatoxin analysis. For peanuts this has been determined (12) to be almost 90% of the total standard deviation in many cases.

Methods of Analysis for Aflatoxins in Peanuts and Peanut Products

Much of the original research on analytical methods used peanuts and peanut products for a number of reasons: Peanut meal as a component of an animal feed caused the initial problem that led to the discovery of aflatoxins, relatively large amounts of peanuts are consumed by children in confections and as peanut butter, and peanut meal was being considered as a protein supplement for use in developing countries by WHO. In the United States and other countries, an industry conscious of the sensitive public relations aspect of a carcinogen in its products cooperated with government agencies to initiate testing and certification systems. Research to develop rapid, practical methods has been stimulated by the large number of samples analyzed annually by governments and industry. But within-lot analytical reliability seems to be limited primarily by the lot sampling system used.

Screening Methods

Early detection and diversion of small lots of contaminated materials may prevent adulteration of much larger accumulations. Screening methods should be capable of detecting as low a level of aflatoxins as the ultimate quantitative methods. Theoretically, screening methods should have a zero possibility of showing false negatives. Dickens and Welty (50) noted a high correlation between aflatoxin content and presence of mold resembling *A. flavus*, readily observable under low magnification in loose or damaged peanut kernels. The USDA grading system for in-shell peanuts requires an actual count of the number of loose and damaged kernels in the sample. As a result of the Dickens and Welty finding, examination of loose or damaged kernels for *A. flavus* mold was introduced into the U.S. peanut inspection system (51). Each inspector has colored photomicrographs of typical peanut molds for guidance. The operation is said to take less than 1 min/sample. Lots of in-shell peanuts having visible *A. flavus* are rejected for all food use except oil milling.

In recent years, improvements in the application of small chromatographic columns, first proposed by Holaday (52), have resulted in reliable and practical methods for screening purposes. The results of a collaborative study of 3 of these methods applied to corn (53) encouraged Romer to develop a broadly applicable minicolumn method (54) based upon the Velasco cleanup (55) and column packing technique (56). The sample is extracted in a blender with acetone-water (85+15) and the filtrate is treated with ferric chloride and copper carbonate to remove plant pigments. The aflatoxin is transferred to chloroform, which is washed with an alkali solution to affect further purification. The chloroform extract is applied to the top of a minicolumn containing successive zones of neutral alumina (top), silica gel, and Florisil (bottom), with calcium sulfate drier at both ends. The column is developed with chloroform-acetone, trapping the aflatoxins as a tight band at the top of the Florisil layer where they can be detected by their blue fluorescence under UV light. By comparison with a previously prepared column containing a known amount of aflatoxin, a decision can be made whether the sample contains more or less aflatoxin than the standard. Aflatoxins, however, are not stable to continued exposure to UV light, making repeated use of such standards unreliable. An advantage of this screening test is that the remaining chloroform extract is sufficiently pure to be used for a TLC presumptive test, as well as for a confirmatory test, should the screening test prove to be positive. The *within-laboratory* precision of this method for total aflatoxins in mixed feeds was estimated to be 10–15% at 20 μg/kg (54).

The Romer screening method was subjected to a collaborative study by Romer and Campbell (57). Thirty-two collaborators from 10 countries examined 11 different agricultural and food products, including peanuts, peanut butter, and peanut meal, in a staggered design at 3 levels of total aflatoxins content: nondetectable (zero), a low level (5–10 μg/kg), and a high level (10–25 μg/kg). Considering only the 4

peanut and peanut product samples, 94% of the laboratories correctly identified the negative samples, 60% the 5 μg/kg samples, 95% the 10, and 100% the 20. The method was recommended for adoption as official first action by the AOAC for peanuts, peanut butter, and peanut meal at total aflatoxins level of 10 μg/kg and above.

Conclusion. — The collaboratively studied Romer minicolumn method, accepted by the AOAC (21, **26.A01–26.A08**) and by the American Association of Cereal Chemists (AACC) (58, Method 45-10), is recommended as a screening method at levels of 10 μg/kg and above in peanuts and its products. It is also applicable to numerous other commodities and to mixed animal feeds. It is a method which may prove very suitable for use by developing countries for monitoring their food supply and for export control.

Presumptive Methods

The first aflatoxin method to be subjected to a collaborative study and adopted by the AOAC (59, 60) was the Celite method. This method was developed in the laboratories of the U.S. FDA and USDA after a critical study (61) of the various methods which had been suggested initially for the analysis of aflatoxin in peanut products. When Lee (62) showed that the addition of water to a peanut meal being extracted with chloroform resulted in more effective removal of aflatoxins, that information was combined with the knowledge which had accumulated within FDA on the use of silica gel columns for the chromatographic separation of aflatoxin from extracted interferences, to produce what is now known as the CB method (63). It became the second method for the determination of aflatoxin in peanut products adopted by the AOAC after a successful collaborative study (64). As peanut butter manufacturers became more involved in testing their products for the possible presence of aflatoxins, they searched for adaptations which would simplify the task of their control laboratories and reduce solvent costs. Waltking *et al.* (65) found that a separatory funnel was simpler and faster for liquid-liquid partition than the Celite column, and centrifugation was faster than filtration for solid separation, thus producing the BF method. When compared with the 2 official AOAC methods in a collaborative study (66), it was found as effective as either one down to a total aflatoxins concentration of 20 μg/kg. The BF method replaced the Celite method as an official AOAC method (67).

The original collaborative study of the CB method (64), using naturally contaminated and spiked peanut butter and naturally contaminated peanut meal, showed an overall recovery of total aflatoxins at and above 30 μg/kg of about 100% and a coefficient of variation of about 35% with 13 collaborators. A series of blind triplicates (3 identical samples spiked at a total aflatoxins level of 80 μg/kg and submitted unknown to each collaborator) showed overall averages of 74, 82, and 80 μg/kg and coefficients of variation of 31, 38, and 43%, respectively. The variation exhibited here is a combination of sample preparation, analyst, and laboratory. In the 3-method collaborative study (Celite, CB, and BF methods) (66), in which 13 laboratories participated and 17 samples were distributed in a staggered design (i.e., each laboratory examined a selected number of the 17 samples), all of the methods were found to be equivalent in accuracy and precision, with about a 70% recovery of added aflatoxin and an overall coefficient of variation of about 35% for total aflatoxins levels down to about 20 μg/kg.

Stack (68) carried the study of the CB and BF methods down to the 2–10 μg/kg range, with 7 collaborators. Both methods proved to be as *accurate* (about 80% recovery) for total aflatoxins at the 5 and 10 μg/kg levels as in the 10–100 μg/kg range covered in the original studies. The CB method is as precise (overall coefficient of variation of about 30%) at the lowest level of 2 μg/kg as at the higher levels. The BF method, which does not provide as good a cleanup as the CB method, loses precision at a total aflatoxins level of about 3 μg/kg. The coefficients of variation of the blanks by both methods are of the order of magnitude of 100%. Analysts examining their plates both visually and densitometrically sometimes obtain values as high as 2 μg/kg on a presumably aflatoxin-free peanut butter. Recoveries at and below the 3 μg/kg level are usually greater than 100% either because of the possible presence of some aflatoxin in the raw material or because of the presence of some interfering material which cannot

Received July 16, 1976.

be distinguished from aflatoxins at these low levels. Figure 3 shows a plot of the overall coefficients of variation (pooled when nonsignificant variables were reported separately) of total aflatoxins by method (when part of the study) for the collaborative studies of the BF and CB methods. There did not appear to be any difference between methods by product. The only significant difference is between methods at the lowest levels, where the BF method has an unacceptable variability for total aflatoxins below 5 μg/kg.

The remarkable showing of the BF and CB methods at such low levels as 5 μg/kg in the latest study may be the result of many years of experience on the part of the participating laboratories, meticulous attention to the chromatographic properties of the silica gel, improved reference standards, and recognition of the laboratory environment as a factor in producing satisfactory TLC plates for visual or instrumental measurement.

A collaborative study for aflatoxin B_1 only, in peanut butter, using 2-dimensional TLC to move background interference from the aflatoxin area, was conducted by Beljaars *et al.* (49). A limit of detection of 3 μg/kg and a coefficient of variation of about 35% at the 3 and 6 μg/kg levels of aflatoxin B_1 were found. This study is not comparable to the collaborative studies described above since the Beljaars study was conducted for aflatoxin B_1 only, using a *single extract pool* from an aflatoxin-free defatted and extracted peanut butter. The extract was used direct (zero value) or spiked. This study therefore measures only the variability associated with the separation and determinative steps.

As a basis for the selection of analytical methods to be recommended for use in France, Jemmali (69) performed 2 separate method comparison collaborative studies. The first study, involving 11 laboratories, showed that estimation of aflatoxin on a TLC plate by comparison with a standard spot was more accurate and precise than estimation by dilution to extinction. The standard spot method with aflatoxin B_1 gave values approximately twice as high as the dilution method (1050 vs. 500 μg/kg) and pooled coefficients of variation of 51 and 77%, respectively. In the second study, the AOAC cottonseed (21, **26.048–26.056**), Celite (70), BF (21, **26.020–26.024**), and CB (21, **26.014–26.019**) methods were compared by 12 laboratories, using naturally contaminated peanut meal and wheat flour. The resulting recommendation of the CB method was based on the low level of background interference in the final extract, achievement of the best precision of the 4 methods, and the simplicity of the required equipment. A further collaborative study by 11 laboratories of the CB method applied to naturally contaminated peanuts and peanut meal gave a pooled between-laboratory coefficient of variation of 16% for aflatoxin B_1 at the mean determined levels of 500 and 750 μg/kg.

As mentioned earlier, some countries, including the United States, Canada, Sweden, and the Federal Republic of Germany, require reporting total aflatoxins ($B_1+B_2+G_1+G_2$). This involves separation and quantitation of the individual aflatoxins. Other countries, especially in the European Economic Community, report only aflatoxin B_1. Both systems have advantages and disadvantages. Most collaborative studies permit comparison of the accuracy and precision of the results reported both ways. In practically all cases, the accuracy and precision data are so similar that the same interpretation of the suitability of a method would be made by either criterion. Quantitation of a single aflatoxin (B_1) is simpler and faster than estimating 4 compounds, which must be cleanly separated from each other as well as from background. Aflatoxin B_1 is the most commonly occurring of the aflatoxins and is usually present in the greatest amount. However, in some commodities, aflatoxin G_1 can be found in amounts exceeding B_1 at an incidence of up to 10% of the samples (71). In rare instances the ratio of G_1 to B_1 can reach 20:1. Aflatoxins B_2 and G_2 are always found in lesser amounts than B_1 or G_1. However, the toxicological aspect is of greatest significance. There is little comparative information on the relative *chronic* toxicity of the aflatoxins; practically all of the comparative studies are of *acute* toxicity. Reporting only aflatoxin B_1 ignores the contribution of the other compounds; reporting total aflatoxins probably overemphasizes the contribution of the other 3 compounds.

Interesting information on the *performance of laboratories* as distinguished from the adequacy of methods for aflatoxin assay is obtained from 2 International Check Sample studies (72, 73). These programs were initiated in 1971 to pro-

vide laboratories around the world with a basis for judging their analytical competence and to determine the need for quality control among the proliferating numbers of laboratories testing for aflatoxins. The findings should encourage those laboratories whose results deviate from the majority to examine their methods and operating techniques. In the first study with a series of 4 samples, in which 129 laboratories participated, a significantly higher mean was found for those laboratories reporting the use of the CB method on peanut products than for those reporting the use of the BF method. In the second study, consisting of a single sample analyzed by 117 laboratories, no difference was found between the mean values for those laboratories using either of the 2 methods. However, in both studies, the coefficients of variation were about twice as great as in the basic collaborative studies upon which official AOAC approval had been granted. This attests to inadequacies in many laboratories from various potential sources, such as not following the method, faulty operating conditions, and improper reference standards. It strongly demonstrated the need for a check sample service.

The International Agency for Research on Cancer in Lyon, France, supported by grants and technical advice from a number of sources, has agreed to provide an international check sample service on a continuing basis. It must be emphasized that since check sample studies do not specify or control the method of analysis to be used in the examination of samples, they are not collaborative studies of methods. The service only supplies reasonably homogeneous samples and ultimately a statistical analysis of the results obtained by the participating laboratories. The responsibility for reviewing the results and making any necessary changes in operating procedures rests entirely with each individual laboratory. Participation in such check sample programs should be encouraged as an important aspect of good laboratory practice.

Conclusion.—The collaboratively studied CB (21, **26.014–26.019**) and BF (21, **26.020–26.024**) methods can be recommended as referee methods for peanuts and peanut products for aflatoxins levels of 5 μg/kg or greater, with a coefficient of variation of about 30%. These methods also have the advantage of applicability, with appropriate modifications, to numerous other aflatoxin-contaminated products. The CB method is also endorsed by the International Union of Pure and Applied Chemistry (IUPAC) (74) and the BF method has been adopted by the American Oil Chemists' Society (AOCS) (75, Method Ab 6-68 (revised 1973)) for peanuts and peanut products.

Confirmation Methods

The possible occurrence of compounds that have a fluorescence and chromatographic behavior similar to the aflatoxins has dictated the need for additional confirmatory tests. This is particularly true when applying the presumptive tests to new commodities and situations and when proof of identity is required for legal and regulatory purposes.

The original chemical test for confirmation of identity was based on the formation of 2 separate derivatives of either aflatoxin B_1 or G_1, using the material isolated by preparative TLC. This can be supplemented by the development of characteristic terata in the embryos observed in the fertile chicken egg bioassay. The U.S. FDA has tested the identity of aflatoxin B_1 isolated from more than 500 samples of contaminated commodities, with 100% coincidence of the chemical and biological test results.

Andrellos and Reid (76) developed the original confirmatory test for aflatoxins B_1 and G_1, using the enol ether function of the difuran moiety. The acid-catalyzed addition products that were formed had characteristic mobilities and fluorescent properties on thin layer chromatograms. The original reagents were acetic acid-thionyl chloride and trifluoroacetic acid which produced, respectively, a dimeric acetate and an addition product with water (aflatoxin B_{2a}). Pohland *et al.* (77) simplified preparation of the epimeric acetates using hydrochloric acid and acetic anhydride and of the water adduct using hydrochloric acid. Both tests were studied collaboratively by Stoloff (78, 79) and both were adopted by the AOAC.

Przybylski (80) further improved the Andrellos test of water adduct formation by eliminating the preparative chromatography, forming the derivative from as little as 0.5 ng aflatoxin B_1 or G_1 directly on the origin spot of the TLC plate. He treated the original spot with trifluoroacetic acid, removed the trifluoroacetic acid by evaporation, developed the chromatogram,

and examined the plate under UV light. A supplementary test is obtained by spraying the developed plate with sulfuric acid (1+3) (81); the fluorescence of aflatoxins B_1, B_2, G_1, G_2, and M_1 and the derivatives of B_1 and G_1 change from blue or blue-green to yellow. This test is not specific. It can only confirm the absence of aflatoxin, i.e., spots which do not turn yellow are positively not aflatoxin. But many materials react with sulfuric acid to give a yellow spot.

The Przybylski test was studied collaboratively by Stack and Pohland (82). In this study an aflatoxin-free extract of peanut butter prepared by the CB method was used. One portion was retained as a negative sample and aflatoxin B_1 was added to the remaining 2 portions to provide the equivalent of 5 and 15 μg/kg original peanut butter, respectively. The portions were further subdivided to provide 7 negative samples, 16 at 5 μg/kg, and 17 at 15 μg/kg, respectively, a total of 40 subsamples. These 40 subsamples were randomly distributed to 8 collaborators. None of the 5 collaborators who received the 7 blanks misidentified the blanks by either the derivative or sulfuric acid spray test. None of the 8 collaborators who received the seventeen 15 μg/kg samples failed to correctly identify the aflatoxin B_1 by either test. One of the 8 laboratories receiving the 5 μg/kg samples failed to identify, by the water adduct test, all 3 such samples distributed to it, but correctly identified all 3 by the sulfuric acid test. Two laboratories missed 3 of four 5 μg/kg samples by the sulfuric acid test, although correctly identifying all 4 samples by the water adduct test. As a result of this study, the method was adopted by the AOAC.

Any extract suitable for presumptive quantitation, regardless of the method by which it is obtained, is usually sufficiently pure to use for confirmation of identity by derivative formation, either after preparatory isolation or directly on the TLC plate. Adjustment of the TLC solvent may be necessary as discussed under the basic steps in the prior section *Chemical Assay, General.*

The only derivatization method for aflatoxins B_2 and G_2 thus far suggested (83) requires too large an amount of the compound to be of practical use.

Conclusion. — The derivative confirmation method on the isolated aflatoxin as described by Pohland (21, **26.066–26.072**) and the Przybylski method of derivative formation on the plate as studied by Stack (21, **26.A17**) are recommended as referee methods for the confirmation of aflatoxins B_1 and G_1 in peanuts and peanut products, and for all other commodities discussed subsequently for which presumptive methods are recommended as referee methods. The cleaned up extract from the Romer method (21, **26.A01–26.A08**) is also suitable for application of the 2 confirmatory tests (54). Variation in TLC composition to adjust for differences in TLC conditions is permissable.

Methods of Analysis for Aflatoxins in Cottonseed and Cottonseed Meals

Aflatoxin contamination of cottonseed is generally characterized by the absence of aflatoxins G_1 and G_2. Cottonseed contains the characteristic polyphenolic pigment gossypol, in addition to the common plant pigments such as chlorophyll, xanthophylls, and flavones. These pigments, which could interfere in the determination of aflatoxins, are removed by precipitation with a heavy metal ion such as lead or zinc, or by adsorption to a ferric gel.

Screening Methods

Marsh *et al.* (84–86) observed in cotton fibers under longwave UV light a glowing bright greenish-yellow (BGY) fluorescence associated with *A. flavus* rot of cotton bolls. The fluorescent compound has been identified as a product of the action of peroxidase on kojic acid, a major metabolite of *A. flavus* and *A. parasiticus*, the species that produce aflatoxins. Rains can leach out the fluorescent compound, which is water soluble, and seeds contaminated after destruction of the peroxidase, a labile enzyme, will not exhibit the fluorescence. Whitten (87) proposed using these observations as a simple screening test for detecting the presence of aflatoxins. He correctly identified 90% of 2300 samples with detectable aflatoxins. False negative identifications occurred only with samples containing less than 10 μg total aflatoxins/kg. The observations, which require no special skill, can be performed rapidly.

Although a number of simplified and rapid methods have been developed specifically for cottonseed and cottonseed products (88–90), the

only one which has been studied collaboratively is the general minicolumn method of Romer (54) which incorporates the extraction solvent and the ferric gel purification step of the presumptive cottonseed methods. It was found to be applicable to cottonseed products, with a detection limit for total aflatoxins of 10 μg/kg. The collaborative study of the method (57) included 3 cottonseed meals. The negative sample, which actually contained confirmed aflatoxin B_1 at 1 μg/kg, a barely detectable level, was identified as positive by 4 and negative by 8 collaborators. Nine of 10 collaborators correctly identified the 10 μg/kg sample and all 12 correctly identified the 20 μg/kg sample.

Conclusion. — The collaboratively studied Romer minicolumn method, accepted by the AOAC (21, **26.A01–26.A08**) and the AACC (58), is recommended as a screening method for total aflatoxins in cottonseed and cottonseed products at a level of 10 μg/kg and above, as well as for numerous other agricultural commodities.

Presumptive Methods

Cottonseed products, particularly ammoniated cottonseed and cottonseed meals, contain some difficult-to-remove interferences. The original AOAC method (21, **26.048–26.056**; 91) utilized an aqueous acetone solvent to obtain an extract essentially free from lipids, followed by precipitation of the gossypol pigments as the lead derivatives, partition into chloroform, further cleanup on a silica gel column, and separation and quantitation by TLC. The collaborative study (92) involved 12 laboratories with 10 samples each, 7 spiked and 3 naturally contaminated. The average recovery of total aflatoxins (B_1+B_2) was 85% by visual and 90% by densitometric estimation, with the unusually low overall coefficients of variation of 18 and 12%, respectively, for samples with aflatoxins levels down to 30 μg/kg. Figure 4 contains a plot of the coefficients of variation as a function of the aflatoxin concentration. The 3 points interconnected by solid lines are data from blind triplicates. The open circle of a pair connected by a dashed line is the densitometric result; the solid circle, the visual result. In this study, the coefficients of variation were essentially the same for between- and within-laboratory error components by either visual or densitometric measurement, reflecting little, if any, bias in the method.

The original official method was simplified and shortened (93) by eliminating the time-consuming steps of boiling after lead salt treatment, by substituting a simple filtration through silica gel and alumina for column chromatography, and by making silica gel chromatography optional except for those samples, such as ammoniated cottonseed products, which contain the most difficult-to-remove interferences. In the collaborative study of the simplified method (94), 11 laboratories analyzed an aflatoxin-negative meal and 12 contaminated samples with total aflatoxins contents of 7–266 μg/kg. Only 1 laboratory reported a false positive for the negative meal. The mean overall coefficients of variation for the contaminated meals, meats, and ammoniated meals were 29, 37, and 38%, respectively, as shown in Fig. 4. The between-laboratory variance is larger than the within-laboratory variance, indicating a systematic error for this method.

Conclusion.—The official AOAC method (21, **26.048–26.056**) with its good precision is recommended as a referee method. The official rapid method (21, **26.A09–26.A16**), which is considerably faster than the original official method and which yields precision estimates consistent with AOAC-approved methods for aflatoxins in other products, can also be recommended as a referee method down to 10 μg/kg.

Methods of Analysis for Aflatoxin in Copra, Copra Meal, and Coconut

The analysis of coconut products for total aflatoxins is difficult because of the presence of interfering materials in the aflatoxin B_2, G_1, and G_2 region.

Screening Methods

Although coconut meal was one of the commodities studied by Romer (54), coconut products were not included in his collaborative study. Therefore no referee screening method is recommended for this commodity.

Presumptive Methods

Baur and Armstrong (95) conducted a collaborative study of a slightly modified CB method for aflatoxins, in which 19 laboratories participated, utilizing 2 copra samples, 1 naturally

contaminated and the other negative, and 4 naturally contaminated copra meals. Quantitative measurements for aflatoxins B_2, G_1, and G_2 could not be made reliably because of background interference, but precision values for B_1 are comparable to those obtained with other commodities, with a coefficient of variation of about 35% down to 35 μg/kg, the lowest level tested.

Conclusion.—The official AOAC method (21, **26.032–26.036**) is recommended as a referee method for the determination of aflatoxin B_1 in coconut products at levels down to 35 μg/kg. This method is also approved by IUPAC (96) and by the AOCS (75, Method Ah 1-72).

Methods of Analysis for Aflatoxins in Corn

Corn contains less material interfering with aflatoxin analysis than any of the other grains except rice. The extracts from white corn contain less interfering material than extracts from yellow corn.

Screening Methods

Shotwell *et al.* (97) described a rapid screening method for corn kernels for possible aflatoxin contamination based upon the presence, when viewed under longwave UV light, of the BGY fluorescence, similar to that seen in aflatoxin-contaminated cottonseed (84). For maximum detectability, the corn must be cracked to expose BGY fluorescence hidden under the seed coat. The method may also be applicable to wheat, oats, and barley (98), but it has not yet been field tested on these grains. A 5 lb (2 kg) sample can be examined in less than 5 min. The test is obviously only indicative of the growth of *A. flavus* in the fluorescing kernel. Positive observations should be followed by a chemical test for aflatoxins.

For rapid screening of corn extracts, Dantzman and Stoloff (99) shortened the CB method by applying the oil residue left after evaporation of the extracting chloroform directly to a TLC plate. The oil is separated from the aflatoxins by preliminary development with anhydrous ether. A second development with one of the usual solvent combinations separates the aflatoxins from remaining interferences. By comparison with reference standards, a rapid estimation of quantity can be made. This method was part of the collaborative study (100) of the application of the CB method to corn and soybeans. This rapid method was found applicable to corn at and above the lowest total aflatoxins level tested, 10 μg/kg.

Shotwell and Stubblefield (53) performed a collaborative study on 3 minicolumn screening methods for aflatoxins in corn: the Pons *et al.* method (90), the Shannon *et al.* modification of the Pons *et al.* method (101), and the Velasco method (56). The Shannon *et al.* modification was applicable to levels of as little as 10 μg total aflatoxins/kg corn and was considered preferable to the original Pons *et al.* method. The Velasco method gave only 1 incorrect detection in 60 assays by 10 collaborators as compared to 4 incorrect detections by the Shannon *et al.* method; the Velasco method was applicable to total aflatoxins as low as 5 μg/kg, but took considerably longer to perform (45 min as compared to 20 min). Both methods were adopted by the AOAC. Selected features of the 3 minicolumn methods were combined by Barabolak *et al.* (102) and by Holaday and Lansden (103), but these combinations have not been subjected to collaborative studies.

Romer also combined various published extraction and minicolumn techniques to obtain a method (54) applicable to mixed feeds and feed ingredients. The general collaborative study of this method (57) established its applicability to the detection of total aflatoxins in white and yellow corn at levels of 10 μg/kg and above.

Conclusion.—Four referee screening methods are recommended for aflatoxin in corn. The minicolumn methods do not require laboratory facilities for their performances. Each method has unique advantages. The Romer method, which has been adopted by the AOAC (21, **26.A01–26.A08**) and the AACC (58) is useful as a general purpose method. The Dantzman and Stoloff rapid screening method (99), also adopted by the AOAC (21, **26.038**) and the AACC (58, Method 45-05), can be conducted as part of the CB presumptive method (21, **26.037–26.039**). The Shannon *et al.* method (21, **26.040–26.043**) can be completed in the least time, and the Velasco method (21, **26.044–26.047**) has the lowest limit of applicability (5 μg/kg) by collaborative test. Both of the latter methods also have been accepted by the AACC (58, Methods 45-15, 45-16).

Presumptive Methods

Shotwell and Stubblefield (100) performed a collaborative study of the CB method applied to corn and soybeans. Fifteen collaborators analyzed 8 corn samples with a recovery in 2 spiked samples of about 70% total aflatoxins (B_1+G_1). The coefficient of variation for those samples containing total aflatoxins of 20 μg/kg or more was about 30%; in the 5–10 μg/kg range, the coefficient of variation was about double this value.

Conclusion.—The CB method, as approved by the AOAC (21, **26.037–26.039**) and by the AACC (58, Method 45-05) is recommended as the referee method for total aflatoxins in corn at levels of 20 μg/kg and above.

Methods of Analysis for Aflatoxins in Tree Nuts

Although for most purposes tree nuts are considered as a class, for aflatoxin analysis each type of nut must be examined individually. For example, pistachio nuts show TLC interferences when extracts are prepared by the BF method, often as red spots which appear variably in areas of the B_1 and G_2 spots (104). For an unknown reason, the G aflatoxins in brazil nut and filbert extracts may be retained on the silica gel column used in the CB method (L. Stoloff & V. DiProssimo, 1972–1973).

Screening Methods

All of the 14 participants in the collaborative study of the Romer method (57) correctly identified the presence of aflatoxins in pistachio nuts at the lowest level of total aflatoxins used, 10 μg/kg. No problems were encountered during the developmental work on this method (54) with brazil nuts, cashews, walnuts, pecans, almonds, and filberts at total aflatoxins levels of 5 μg/kg. Therefore the collaboratively studied Romer method (21, **26.A01–26.A08**) is recommended as a screening method for tree nuts at 10 μg/kg and above.

Presumptive Methods

DiProssimo (104) conducted a collaborative study of the CB and BF methods with pistachio nuts, using both naturally contaminated and spiked samples containing total aflatoxins at levels of 15–57 μg/kg. The statistical design included "blind" triplicates with both types of contamination to permit a separation of the between- and within-laboratory components of error. The CB method recovered more aflatoxin than the BF method, approximately 85 vs. 65%, respectively. The coefficient of variation was about 40% for both methods at the total aflatoxin levels of 35–60 μg/kg, remaining at about the same level (50%) for the CB method at 15 μg/kg but increasing to an unacceptable 70% with the BF method for the same sample. The BF method also gave more false positive results with negative samples than the CB method, reflecting the fact that with most commodities the CB method gives a cleaner exract than the BF method. Operationally, the BF method is considerably faster and more economical than the CB method.

The coefficient of variation for each method is plotted as a function of concentration in Fig. 5. For this figure, the original data were used to recalculate coefficients of variation, where necessary, to provide the same basis as plotted in the previous figures, so that a direct comparison of the data among commodities is possible. The design of this study also permitted the calculation of the standard deviation of a single analysis by a single laboratory (analyst), which, when expressed as a coefficient of variation, is about 40%, approximately the same as the total overall variability. The standard deviation of a single analysis is interpreted as follows: The "true" aflatoxin content of a sample analyzed by a single determination by a given laboratory will lie, 95% of the time, within the range determined by the value found ±2 times the standard deviation.

Conclusion.—The official AOAC CB (21, **26.063**) and BF (21, **26.064**) methods are recommended as referee methods for the determination of total aflatoxins in tree nuts down to 35 μg/kg.

Methods of Analysis for Aflatoxins in Soybeans and Small Grains

Soybeans and the small grains (wheat, oats, rye, sorghum, and barley) usually do not present an aflatoxin contamination problem, either because of the possible presence of an inhibitory substance, as in the case of soybeans, or because the conditions of seed development and maturation are not conducive to mold growth, as in the case of small grains.

Screening Methods

Because of the infrequency of aflatoxin contamination in soybeans and small grains in the U.S., these commodities were not included as such in the 2 Romer studies (54, 57). However, soybean meal constituted a substantial part of the mixed feeds included in these studies. The incidence of false negatives for 4 mixed feeds was very low at a total aflatoxins level of 25 μg/kg. Therefore, the collaboratively studied Romer method (21, **26.A01–26.A08**) is recommended as a screening method for soybeans at 25 μg/kg and above.

Presumptive Methods

Shotwell and Stubblefield (100) performed a collaborative study of the CB method on both corn and soybeans. Thirteen collaborators analyzed 7 soybean samples with a recovery in 2 spiked samples of about 70% total aflatoxins (B_1+G_1). The coefficient of variation for samples containing total aflatoxins at 20 and 60 μg/kg, naturally contaminated and spiked, was a high 45%, with an unexplained spread of the coefficients of variation in an identical series of triplicates of 64, 52, and 20%, as shown in Fig. 6. The low incidence of aflatoxin in this commodity has created no urgency to investigate the cause of these large variations. Although the AOAC (21, **26.065**) and the AACC (58, Method 45-05) have accepted the CB method for soybeans, it appears to have too great a variation for acceptance as a referee method.

Methods of Analysis for Aflatoxin in Cocoa Beans

The usual extracting solvents and partitioning systems are excellent solvents for theobromine from cocoa beans and its products. The sheer bulk of this compound interferes with the isolation of the aflatoxins. Although cocoa beans were on the original list of suspect commodities because of the climatic and environmental situation and usual agricultural practices under which they are produced, aflatoxin contamination of cocoa beans has not been demonstrated. No evidence has appeared to warrant additional work to improve present methods for aflatoxin in this product.

Screening Methods

Collaboratively studied screening methods have not been applied to cocoa products.

Presumptive Methods

Scott (105) was able to apply the CB method to cocoa beans by removing most of the lipids with hexane before extracting with chloroform, and then removing most of the theobromine with silver nitrate. In the collaborative study utilizing 11 laboratories (106) on samples of cocoa beans containing added total aflatoxins at 45 and 70 μg/kg, the average recovery obtained was 85% with a coefficient of variation of 35%. At 18 μg/kg the coefficient of variation approached an unacceptable 50%. These data are shown in Fig. 7.

Conclusions. — The collaboratively studied Scott modification of the CB method (21, **26.025–26.031**), which has also been accepted by IUPAC (107), is recommended as the referee method for cocoa beans at total aflatoxins levels of 45 μg/kg and above.

Methods of Analysis for Aflatoxins in Green Coffee

The problem with the analysis of green coffee beans for aflatoxin is similar to that with cocoa beans—caffeine and interfering fluorescent materials are extracted and must be removed. No aflatoxin was found in a survey of 58 samples of moldy coffee from various countries (108).

Screening Methods

Collaboratively studied screening methods have not been applied to coffee.

Presumptive Methods

Levi (109) applied the CB method as modified by Scott (110) to coffee beans by grinding the beans, after freezing, in a blender. The chloroform extract was cleaned up on a deactivated Florisil column; impurities were eluted with tetrahydrofuran. In the collaborative study, the results from 8 laboratories showed a 70% overall recovery of added aflatoxin B_1, but the coefficient of variation of 3 sets of duplicate samples was almost 50% with aflatoxin B_1 at 25 and 80 μg/kg. There were no false positives with a negative sample, and 6 of the 8 laboratories detected the presence of aflatoxin B_1 at 5 μg/kg. The variability of results by this method, as shown in Fig. 7, is too great for referee use.

Conclusion.—No recommendation can be made for a referee method for the detection, determination, or confirmation of aflatoxin in coffee beans.

Methods of Analysis for Aflatoxins in Mixed Feeds

Mixed feeds, particularly those containing alfalfa, corn gluten, and fish meals (111), present a difficult separation problem.

Screening Methods

The Romer method (54) was specifically developed for mixed feeds. It successfully handled commercial chick, pig, turkey starter, and dairy rations in the collaborative study (57) at total aflatoxins levels of 15 μg/kg and above.

Presumptive Methods

The Commission of the European Communities is reported to have conducted a collaborative study on straight and mixed feeds, using a 2-dimensional TLC method. Citrus pulp presents difficulties in the analysis. The results of the collaborative study have not been published. Therefore no method can be recommended as a referee method for *mixed feeds*, although methods are available for the individual ingredients, as discussed under the specific agricultural commodities.

Conclusion.—Only the Romer screening method (21, **26.A01–26.A08**) can presently be recommended for the examination of mixed feeds at total aflatoxins levels of 15 μg/kg and above.

Methods of Analysis for Aflatoxins in Milk Products and Animal Tissues

The methodology developed for studies of aflatoxin metabolism required limits of detection 2 orders of magnitude lower than the concentration in the feed ingredient. These methods have been adapted to analysis of the edible tissues: aflatoxin M_1 in milk, aflatoxin B_1 in eggs, and aflatoxins B_1 and M_1 in meat (particularly liver). The practical limit of detection for aflatoxins in these tissues is about 0.1 μg/kg (wet weight), although under research conditions the limit has been pressed to around 0.02 μg/kg for aflatoxin M_1 in milk and aflatoxin B_1 or M_1 in liver.

The primary metabolite found in milk is aflatoxin M_1, with M_2 sometimes observable as a minor constituent. Under research dosing conditions, aflatoxin B_1 may also be seen as a minor constituent. Separation of aflatoxin M_2 from M_1 on silica gel TLC plates is difficult. Therefore, as a practical matter for survey purposes, aflatoxins M_1 and M_2 are measured together.

Only aflatoxin B_1 has been found transmitted to eggs; both B_1 and M_1 are found in liver.

Screening Methods

No screening methods have been proposed for animal products.

Presumptive Methods—Milk Products

Stubblefield *et al.* (112) compared the performance of 6 published methods for the determination of aflatoxin M_1 in liquid and powdered milk, using comparison criteria of a detection level of 0.1 μg/kg in fluid milk and 1 μg/kg in powdered milk, and the relative recovery of aflatoxin M_1 from naturally contaminated and spiked samples at fluid milk equivalent levels of 0.1, 0.5, and 1.0 μg/kg. The Purchase and Steyn method (113) was selected as most suitable for assaying powdered milk and the Jacobson *et al.* method (114) for liquid milk. On the basis of experience gained in this work, these methods were further simplified without sacrificing any of their desirable attributes.

A collaborative study of these 2 methods, involving 15 laboratories, was performed by Purchase *et al.* (115) using the Purchase and Steyn method (113) for powdered milk and the Jacobson *et al.* method (114) for liquid milk. The coefficients of variation found are given in Fig. 8.

With *powdered* milk, 5 of 13 laboratories reported false positives on the negative sample. One false negative was reported by 13 laboratories at the lowest level of aflatoxin M tested, 1.2 μg/kg. The coefficient of variation was 36% at this level. The highest level tested, 50 μg/kg, had the highest coefficient of variation, 47%. Overall, the recovery of "expected" aflatoxin M by this method was 64% with a coefficient of variation of about 30%.

Both spiked and naturally contaminated *liquid* milk samples were used in the collaborative study. For the naturally contaminated samples, the average of the reported values was 85% of the value established by the initiating laboratory by the same method. The recovery from the spiked samples was 47%. The coefficients of variation showed an unusual pattern. The 3 samples naturally contaminated at about 1 μg/kg had the lowest coefficient of variation,

about 37%. The lowest and highest aflatoxin levels, 0.1 and 5.0 μg/kg, had the highest coefficients of variation, about 100%. There were 2 false positive observations in 13 reports on the negative sample and 5 false negative samples at the lowest spiked level, 0.1 μg/kg.

Pons *et al.* (116) developed a method applicable not only to milk but to its heat- and acid-processed products (concentrated milks and cheeses). The aflatoxin is extracted with acetone-water, phospholipids and soluble proteins are removed with lead acetate, and residual fats are partitioned into hexane. Aflatoxin M is then removed with chloroform, which is washed with salt solution. For fluid milk, the residue after evaporation of the chloroform may be used directly for TLC. Heat- and acid-treated milk products must be further purified by partition chromatography on a cellulose-aqueous methanol column prior to TLC. This method can be performed more easily and rapidly than the 2 methods previously tested and is more generally applicable. It was further refined by Stubblefield and Shannon (117) and then subjected to a collaborative study involving 19 international participants (118). Samples were prepared by spiking the milk prior to preparation of each milk product and also by spiking specific sample portions immediately before shipping. The coefficients of variations are large (45–75%) at these low levels of aflatoxin M_1 (0.1–3 μg/kg), using milks (liquid and dried), butter, and cheese (ricotta, cheddar). A negative blue cheese gave a number of false positives because of the presence of a fluorescent interference in the aflatoxin M_1 area.

Schuller *et al.* (45) performed a collaborative study of the determination of aflatoxin M_1 in naturally contaminated liquid and powdered milk, utilizing a Celite isolation procedure (59) and 2-dimensional TLC (119). Eight samples (4 pairs of blind duplicates) were examined by 21 laboratories. No negative samples were included. For this paper, the original data have been reanalyzed statistically to permit comparing the coefficients of variation with that of Purchase (115) as given in Fig. 8. The liquid milk contaminated with the lowest level, 0.05 μg aflatoxin M/L, showed almost 20% false negatives. At the higher level, 0.16 μg aflatoxin M/L, the coefficient of variation was about 50% with no false negatives. The coefficient of variation for powdered milk was about 30% for the 2 levels studied, 1.5 and 4 μg aflatoxin M/L.

Conclusion.—At levels of aflatoxins of the order of 0.1–1 μg/kg the problem of separation of the contaminant from the interferences is magnified over that encountered in plant material, resulting in coefficients of variation so high (45–75%) that it is doubtful that meaningful conclusions can be drawn as to the relative performance of methods other than most laboratories are able to determine correctly the presence or absence of the aflatoxin. Even here, confirmation by derivatization is a necessity since the results show about a 10% probability that a false positive or false negative result will be obtained.

A coefficient of variation of 50% on a sample showing an aflatoxin M_1 level of 1 μg/kg, which is regarded as a heavily contaminated milk sample, means that independent samples analyzed by different laboratories will show values of from 0 to 2 μg/kg 95% of the time. A finite proportion of the results, 2.5%, are to be expected as false negatives and 2.5% of the results will be an extremely improbable value (for milk) of greater than 2. These are expected values, derived from the normal operations of laboratories operating under conditions where the laboratories knew that their results would be compared with the results of other laboratories. Routine conditions would be expected to produce a poorer showing. It is therefore necessary to institute an extremely rigorous quality assurance program, as well as to recognize the need for the analysis of a number of independent samples from the same lot, when an individual result is likely to be used as the basis for critical decisions such as in legal actions and for referee purposes.

Nevertheless, since analysts must live with the best which is available, the collaboratively studied methods, despite their limitations, have been accepted by the AOAC and IUPAC: the modified Pons method (21, **26.079–26.083**) for milk products in general, the Purchase and Steyn method for powdered milk (21, **26.088–26.089**; 115), and the modified Jacobson method for fluid milk (21, **26.084–26.087**; 115). In view of the reported variability in results, these methods cannot be recommended as referee methods. Reliance for referee purposes must be associated with the use of several lab-

oratories or numerous samples to reduce the variability to a reasonable range.

Presumptive Methods—Animal Tissue

Methods for aflatoxins in animal tissues have been developed by only a few workers. Rossi *et al.* (120) used 2-dimensional TLC. Brown *et al.* (121) combined the extraction and partition of Jacobson *et al.* (114) with the silica gel column of the CB method and the cellulose column of Pons *et al.* (116), followed by TLC and measurement by fluorodensitometry. Since the *within-laboratory* coefficient of variation of the basic portions of the Brown *et al.* method (121) at the 0.05–0.2 μg/kg levels of aflatoxin are already over 40%, it is unlikely to be suitable as a referee method.

No collaborative studies have been performed using animal tissues.

Confirmatory Methods

Because of the uncertainties involved in determining the low levels of aflatoxin M found in animal tissues and foods of animal origin, the reliability of confirmation tests assumes greater importance. Stack *et al.* (122) described a derivative technique specifically developed for aflatoxin M_1, in which 30 ng aflatoxin is used but as little as 1 ng is detected. Use of such low quantities requires a preliminary cleanup by preparative TLC or by column chromatography. This method was included in the collaborative study performed by Stubblefield and Shannon (118). When an estimated 30 ng aflatoxin M_1 added to milk was carried through the entire method, 5 false negatives were reported in 29 observations on the formation of the 2 derivatives specified in the method. When 50 ng aflatoxin M_1 was added to an extract from the equivalent of 50 g colby cheese, all observations for the acetate derivative were correct, and only 1 false negative was reported for the hemiacetal derivative. The study had no provision for judging the possibility of false positive observations. A modification of the Przybylski method has been developed for aflatoxin M (123), but it has not yet been tested collaboratively.

Conclusion.—The derivative formation and TLC method as approved by the AOAC (21, **26.090**) is recommended as a referee method for confirming the identity of aflatoxin M_1 with quantities greater than 30 ng.

Table 1. Sources of aflatoxin standards (1976)

Source	Compounds available[a]
Aldrich Chemical Co., 940 St. Paul Ave, Milwaukee, WI 53233	aflatoxins
Applied Science Laboratories, Inc., PO Box 440, State College, PA 16801	quantitative B_1, B_2, G_1, G_2 standard kit
Calbiochem, 10933 N Torrey Pines, La Jolla, CA 92037	aflatoxins
Makor Chemicals, Ltd, PO Box 6570, Jerusalem, Israel	aflatoxins
A. E. Pohland, Food and Drug Administration, 200 C St, SW, Washington, DC 20204	aflatoxins
Rijksinstituut voor de Volksgezondheid, PO Box 1, Bilthoven, The Netherlands	aflatoxins B_1 and M_1 qualitative mixture of B_1, B_2, G_1, G_2
Senn Chemicals, CH-8157 Dieseldorf, Switzerland	aflatoxins
R. M. Stubblefield, U. S. Department of Agriculture, 1815 N University St, Peoria, IL 61604	aflatoxins M_1 and M_2
Supelco, Inc., Supelco Park, Bellefonte, PA 16823	aflatoxins

[a] B_1, B_2, G_1, and G_2, unless otherwise specified.

Standards

All of the recommended screening, presumptive, and confirmatory methods for aflatoxins depend upon comparison with a reference standard. The identity and purity of the standards used in conjunction with the performance of the method ultimately determine the success of the analysis. Suitable standards are now internationally available from a number of sources as shown in Table 1. Rodricks (124) recommended that all collaborative studies of quantitative methods for mycotoxins include (a) information on the characterization of the standards, their source, methods of isolation and purification, criteria of purity, and stability under conditions for dispensing and use, and (b) a method for checking concentration and purity of the reference standards. The basic UV absorption characteristics of solutions of crystalline preparations of the 4 aflatoxins, from several laboratories,

Table 2. Collaboratively studied methods of analysis recommended as referee methods

Method/commodity	Min. level applicability[a]	Coeff. of var., %	Ref.	Remarks
Biological method				
Aflatoxin B_1 isolates	2 μg (B_1)	—	AOAC **26.073-26.078** (21)	chicken embryo bioassay for toxicity
Screening methods				report positive or negative
Peanuts, cottonseed, corn, tree nuts, and their products	10	—	AOAC **26.A01-26.A08** (21) AACC 45-10 (58)	Romer minicolumn method
Mixed feeds	15	—		
Soybean meal	25	—		
Corn	10	—	AOAC **26.038** (21) AACC 45-05 (58)	part of CB method
	10	—	AOAC **26.040-26.043** (21) AACC 45-16 (58)	most rapid
	5	—	AOAC **26.044-26.047** (21) AACC 45-15 (58)	lowest limit of applicability
Confirmatory methods				
All extracts suitable for presumptive tests for B_1 and G_1, including that for Romer screening test	20	—	AOAC **26.066-26.071** (21)	Pohland modification
	15	—	AOAC **26.A17** (21)	Przybylski modification
Extracts suitable for M_1 presumptive tests	30 ng (M_1)	—	AOAC **26.090** (21)	acetate/hemiacetal
Presumptive methods				
Peanuts and its products	5	30	AOAC **26.014-26.019** (21) IUPAC Inform. Bull. 31 (74)	CB method
	5	30	AOAC **26.020-26.024** (21) AOCS Ab 6-68 (rev. 1973) (75)	BF method
Cottonseed and its products	30	15	AOAC **26.048-26.056** (21) AOCS Aa 8-71T (75)	original method
	10	30	AOAC **26.A09-26.A16** (21)	rapid modification
Coconut and its products	35 (B_1)	35	AOAC **26.032-26.036** (21) IUPAC Inform. Bull. Tech. Rep. 9 (96) AOCS Ah 1-72 (75)	modified CB
Corn	20	30	AOAC **26.037-26.039** (21) AACC 45-05 (58)	CB method
Tree nuts	35	40	AOAC **26.063** (21)	CB method
	35	40	AOAC **26.064** (21)	BF method
Cocoa beans	45	35	AOAC **26.025-26.031** (21) IUPAC Inform. Bull. Tech. Rep. 8 (107)	modified CB

[a] Applicable to total aflatoxins, μg/kg, unless otherwise indicated as specific compound or as absolute amount.

were determined in 2 laboratories (125). These constants, in turn, were used to determine the concentration of aflatoxin in standard solutions supplied to collaborators in 3 separate studies summarized by Rodricks and Stoloff (126). The average coefficient of variation for the determined concentrations of the 4 aflatoxins in these studies is 4.5%, approaching the limit of spectrophotometric precision. In a related study, Purchase and Altenkirk (127, 128) determined the stability of aflatoxin M_1 standard solutions.

Conclusion.—The criteria of purity and methods for determining the purity and concentration of aflatoxins as approved by the AOAC (21, **26.004–26.013**) and IUPAC (129) should be part of all referee methods.

Summary

Table 2 gives those methods which are judged as suitable as referee methods, by commodity, with appropriate applicability criteria.

References

(1) Food and Agriculture Organization of the United Nations/World Health Organization/United Nations Environment Pro-

gramme (Feb. 1975) Working Paper on Methods of Analysis and Sampling for Aflatoxins, EC/MAS/FC/75/2, Rome, Italy
(2) Hartley, R. D., Nesbitt, B. F., & O'Kelly, J. (1963) Toxic metabolites of *Aspergillus flavus*. *Nature London* **198**, 1056–1058
(3) Robertson, J. A., Lee, L. S., Cucullu, A. F., & Goldblatt, L. A. (1965) Assay of aflatoxin in peanuts and peanut products using acetone-hexane-water for extraction. *J. Am. Oil Chem. Soc.* **42**, 467–471
(4) Cucullu, A. F., Lee, L. S., Mayne, R. Y., & Goldblatt, L. A. (1966) Determination of aflatoxins in individual peanuts and peanut sections. *J. Am. Oil Chem. Soc.* **43**, 89–92
(5) Koltun, S. P., Gardner, H. K., Jr., Dollear, F. G., & Rayner, E. T. (1974) Physical properties and aflatoxin content of individual cateye fluorescent cottonseeds. *J. Am. Oil Chem. Soc.* **51**, 178–180
(6) Shotwell, O. L., Goulden, M. L., & Hesseltine, C. W. (1974) Aflatoxin distribution in contaminated corn. *Cereal Chem.* **51**, 492–499
(7) Whitaker, T. B., & Wiser, E. H. (1969) Theoretical investigations into the accuracy of sampling shelled peanuts for aflatoxin. *J. Am. Oil Chem. Soc.* **46**, 377–379
(8) Whitaker, T. B., Dickens, J. W., & Wiser, E. H. (1970) Design and analysis of sampling plans to estimate aflatoxin concentrations in shelled peanuts. *J. Am. Oil Chem. Soc.* **47**, 501–504
(9) Whitaker, T. B., Dickens, J. W., Wiser, E. H., & Monroe, R. J. (1974) Development of a method to evaluate sampling plans used to estimate aflatoxin concentrations in lots of shelled peanuts. *IUPAC Inf. Bull. Tech. Rep.* No. 10, 16 pp.
(10) Whitaker, T. B., Dickens, J. W., Monroe, R. J., & Wiser, E. H. (1972) Comparison of the observed distribution of aflatoxin in shelled peanuts to the negative binomial distribution. *J. Am. Oil Chem. Soc.* **49**, 590–593
(11) Peanuts—1975 Crop. Incoming and outgoing quality regulations and indemnification. (15 July 1975) *Fed. Regist.* **40**(136), 29739–29746
(12) Whitaker, T. B., Dickens, J. W., & Monroe, R. J. (1974) Variability of aflatoxin test results. *J. Am. Oil Chem. Soc.* **51**, 214–218
(13) Tiemstra, P. J. (1969) A study of the variability associated with sampling peanuts for aflatoxin. *J. Am. Oil Chem. Soc.* **46**, 667–672
(14) Velasco, J., Whitaker, T. B., & Whitten, M. E. (1975) Sampling cottonseed lots for aflatoxin contamination. *J. Am. Oil Chem. Soc.* **52**, 191–195
(15) Whitaker, T. B., Whitten, M. E., & Monroe, R. J. (1976) Variability associated with testing cottonseed for aflatoxin. *J. Am. Oil Chem. Soc.* **53**, 502–505
(16) International Organization for Standardization (1967) Oilseeds-Sampling, ISO/R 542—1967 (E) (available from national standards organizations)
(17) Barnes, J. M. (1970) Aflatoxin as a health hazard. *J. Appl. Bacteriol.* **33**, 285–298
(18) Stoloff, L., Campbell, A. D., Beckwith, A. C., Nesheim, S., Winbush, J. S., & Fordham, O. M., Jr. (1969) Sample preparation for aflatoxin assay: The nature of the problem and approaches to a solution. *J. Am. Oil Chem. Soc.* **46**, 678–684
(19) Stoloff, L., Dantzman, J., & Wegener, J. (1972) Preparation of lot samples of nut meats for mycotoxin assay. *J. Am. Oil Chem. Soc.* **49**, 264–266
(20) Jones, B. D. (1972) *Methods of Aflatoxin Analysis*, Report G70, Tropical Products Institute, 56/62 Gray's Inn Rd, London WC1X 8LU, United Kingdom, 58 pp.
(21) *Natural Poisons* (1975) Reprint of Chapter 26 from *Official Methods of Analysis* (1975) (12th Ed.), revised to include Changes in Methods, *JAOAC*, March 1975. AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20044, 24 pp.
(22) Dickens, J. W., & Satterwhite, J. B. (July 1969) Subsampling mill for peanut kernels. *Food Technol.* **23**, 950–952
(23) Velasco, J., & Morris, A. L. (1976) Use of water slurries in aflatoxin analysis. *J. Agric. Food Chem.* **24**, 86–88
(24) Carnaghan, R. B. A., Hartley, R. D., & O'Kelly, J. (1963) Toxicity and fluorescence properties of aflatoxins. *Nature London* **200**, 1101
(25) Wogan, G. N. (1965) Experimental toxicity and carcinogenicity of aflatoxins. *Mycotoxins in Foodstuffs*, Wogan, G. N. (Ed.), The MIT Press, Cambridge, MA pp. 163–173
(26) Purchase, I. F. H. (1967) Acute toxicity of aflatoxins M_1 and M_2 in one-day old ducklings. *Food Cosmet. Toxicol.* **5**, 339–342
(27) Burmeister, H. R., & Hesseltine, C. W. (1966) Survey of the sensitivity of microorganisms to aflatoxins. *Appl. Microbiol.* **14**, 403–404
(28) Clements, N. L. (1968) Rapid confirmatory test for aflatoxin B_1, using *Bacillus megaterium*. *JAOAC* **51**, 1192–1194
(29) Verrett, M. J., Winbush, J., Reynaldo, E. F.,

& Scott, W. F. (1973) Collaborative study of the chicken embryo bioassay for aflatoxin B_1. *JAOAC* **56**, 901–904

(30) Pons, W. A., Jr., Robertson, J. A., & Goldblatt, L. A. (1966) Objective fluorometric measurement of aflatoxins on TLC plates. *J. Am. Oil Chem. Soc.* **43**, 665–669

(31) Coomes, T. J., Crowther, P. C., Francis, B. J., & Stevens, L. (1965) The detection and estimation of aflatoxin in groundnuts and groundnut materials. Part IV. Routine assessment of toxicity due to aflatoxin B_1. *Analyst* **90**, 492–496

(32) Beckwith, A. C., & Stoloff, L. (1968) Fluorodensitometric measurement of aflatoxin thin layer chromatograms. *JAOAC* **51**, 602–608

(33) Ayers, J. L., & Sinnhuber, R. O. (1966) Fluorodensitometry of aflatoxin on thin-layer plates. *J. Am. Oil Chem. Soc.* **43**, 423–424

(34) Beljaars, P. R., & Fabry, F. H. M. (1972) Quantitative fluorodensitometric measurements of aflatoxin B_1 with a flying spot densitometer. I. Fluorodensitometric study of the behavior of aflatoxin B_1 standard spots on different types of silica gel. *JAOAC* **55**, 775–780

(35) Peterson, R. E., Ciegler, A., & Hall, H. H. (1967) Densitometric measurement of aflatoxin. *J. Chromatogr.* **27**, 304–307

(36) Pons, W. A., Jr. (1971) Evaluation of reflectance fluorodensitometry for measuring aflatoxin on thin layer plates. *JAOAC* **54**, 870–873

(37) Schuller, P. L., Verhülsdonk, C. A. H., & Paulsch, W. E. (1970) Determination of aflatoxin B_1 by two-dimensional thin-layer chromatography with fluorodensitometric evaluation. *Arzneim. Forsch.* **20**, 1517–1520

(38) Andrellos, P. J., Beckwith, A. C., & Eppley, R. M. (1967) Photochemical changes of aflatoxin B_1. *JAOAC* **50**, 346–350

(39) Goldblatt, L. A. (1969) *Aflatoxin: Scientific Background, Control, and Implications,* Academic Press, New York, NY, pp. 77–105

(40) Stoloff, L. (1972) Analytical methods for mycotoxins. *Clin. Toxicol.* **5**, 465–494

(41) Codex Alimentarius Commission, Joint FAO/WHO Food Standards Program (1975) Procedural Manual, 4th Ed., p. 69, Rome, Italy

(42) Fischbach, H., & Rodricks, J. V. (1973) Current efforts of the Food and Drug Administration to control aflatoxins in food. *JAOAC* **56**, 767–770

(43) Allcroft, R., & Carnaghan, R. B. A. (1963) Groundnut toxicity—an examination for toxin in human food products from animals fed toxic groundnut meal. *Vet. Rec.* **75**, 259–263

(44) de Iongh, H., Vles, R. O., & van Pelt, J. G. (1964) Milk of mammals fed an aflatoxin-containing diet. *Nature London* **202**, 466–467

(45) Schuller, P. L., Verhülsdonk, C. A. H., & Paulsch, W. E. (1974) Aflatoxin M_1 in liquid and powdered milk *in Symposium on Mycotoxins in Food,* IUPAC, Pulawy, Poland, in press

(46) Jacobson, W. C., & Wiseman, H. G. (1974) The transmission of aflatoxin B_1 into eggs. *Poult. Sci.* **53**, 1743–1745

(47) Purchase, I. F. H. (1972) Aflatoxin residues in food of animal origin. *Food Cosmet. Toxicol.* **10**, 531–544

(48) Armbrecht, B. (1971) Aflatoxin residues in food derived from plant and animal sources. *Residue Rev.* **4**, 13–54

(49) Beljaars, P. R., Verhülsdonk, C. A. H., Paulsch, W. E., & Liem, D. H. (1973) Collaborative study of two-dimensional thin layer chromatographic analysis of aflatoxin B_1 in peanut butter extracts, using the anti-diagonal spot application technique. *JAOAC* **56**, 1444–1451

(50) Dickens, J. W., & Welty, R. E. (1969) Detecting farmers stock peanuts containing aflatoxin by examination for visible growth of *Aspergillus flavus*. *Mycopathol. Mycol. Appl.* **37**, 65–69

(51) Farmers' Stock Peanuts. Inspection Instructions Revised June 1975, U. S. Department of Agriculture, Agricultural Marketing Service, Fruit and Vegetable Division, Washington, DC 20025

(52) Holaday, C. E. (1968) Rapid method for detecting aflatoxins in peanuts. *J. Am. Oil Chem. Soc.* **45**, 680–682

(53) Shotwell, O. L., & Stubblefield, R. D. (1973) Collaborative study of three screening methods for aflatoxin in corn. *JAOAC* **56**, 808–812

(54) Romer, T. R. (1975) Screening method for the detection of aflatoxins in mixed feeds and other agricultural commodities with subsequent confirmation and quantitative measurement of aflatoxins in positive samples. *JAOAC* **58**, 500–506

(55) Velasco, J. (1972) Modified ferric gel method for determining aflatoxin in cottonseed meals. *JAOAC* **55**, 1359–1360

(56) Velasco, J. (1972) Detection of aflatoxin using small columns of Florisil. *J. Am. Oil Chem. Soc.* **49**, 141–142

(57) Romer, T. R., & Campbell, A. D. (1976) Collaborative study of a screening method

for the detection of aflatoxins in mixed feeds, other agricultural products, and foods. *JAOAC* **59**, 110–117

(58) Revisions to AACC Approved Methods (1975) American Association of Cereal Chemists, 3340 Pilot Knob Rd, St. Paul, MN 55121

(59) Nesheim, S. (1964) Mycotoxins: Studies of the rapid procedure for aflatoxin in peanuts, peanut meal, and peanut butter. *JAOAC* **47**, 1010–1017

(60) Campbell, A. D., & Funkhouser, J. T. (1966) Collaborative study on the analysis of aflatoxins in peanut butter. *JAOAC* **49**, 730–739

(61) Trager, W. T., Stoloff, L., & Campbell, A. D. (1964) A comparison of assay procedures for aflatoxin in peanut products. *JAOAC* **47**, 993–1001

(62) Lee, W. V. (1965) Quantitative determination of aflatoxin in peanut products. *Analyst* **90**, 305–307

(63) Eppley, R. M. (1966) A versatile procedure for assay and preparatory separation of aflatoxins from peanut products. *JAOAC* **49**, 1218–1223

(64) Eppley, R. M., Stoloff, L., & Campbell, A. D. (1968) Collaborative study of "a versatile procedure for assay of aflatoxins in peanut products," including preparatory separation and confirmation of identity. *JAOAC* **51**, 67–73

(65) Waltking, A. E., Bleffert, G., & Kiernan, M. (1969) An improved rapid physiochemical assay method for aflatoxin in peanuts and peanut products. *J. Am. Oil Chem. Soc.* **45**, 880–884

(66) Waltking, A. E. (1970) Collaborative study of three methods for determination of aflatoxins in peanuts and peanut products. *JAOAC* **53**, 104–113

(67) Stoloff, L. (1974) Report on mycotoxins. *JAOAC* **57**, 274–278

(68) Stack, M. E. (1974) Collaborative study of AOAC Methods I and III for the determination of aflatoxins in peanut butter. *JAOAC* **57**, 871–874

(69) Jemmali, M. (1973) Collaborative studies of the determination of aflatoxins in peanut products in France. *Pure Appl. Chem.* **35**, 267–270

(70) *Official Methods of Analysis* (1970) 11th Ed., AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20044, secs. 26.021–26.025

(71) DiProssimo, V. P. (1976) Distribution of aflatoxins in some samples of peanuts. *JAOAC* **59**, 941–944

(72) Coon, F. B., Baur, F. J., & Symmes, L. R. L. (1972) International aflatoxin check sample program: 1971 study. *JAOAC* **55**, 315–327

(73) Coon, F. B., Baur, F. J., & Symmes, L. R. L. (1973) International aflatoxin check sample program: 1972 study. *JAOAC* **56**, 322–327

(74) International Union of Pure and Applied Chemistry (March 1968) Information Bulletin No. 31. Bank Court Chambers, 2/3 Pound Way, Crowley Centre, Oxford OX4 3YF, United Kingdom

(75) The Official and Tentative Methods of Analysis of the American Oil Chemists' Society (revised 1973) American Oil Chemists' Society, 508 S Sixth St, Champaign, IL 61820

(76) Andrellos, P. J., & Reid, G. R. (1964) Confirmatory tests for aflatoxin B_1. *JAOAC* **47**, 801–803

(77) Pohland, A. E., Yin, L., & Dantzman, J. G. (1970) Rapid chemical confirmatory method for aflatoxin B_1. I. Development of the method. *JAOAC* **53**, 101–102

(78) Stoloff, L. (1970) Rapid chemical confirmatory method for aflatoxin B_1. II. Collaborative study. *JAOAC* **53**, 102–104

(79) Stoloff, L. (1967) Collaborative study of a method for the identification of aflatoxin B_1 by derivative formation. *JAOAC* **50**, 354–360

(80) Przybylski, W. (1975) Formation of aflatoxin derivatives on thin layer chromatographic plates. *JAOAC* **58**, 163–164

(81) Schuller, P. L., Ockhuizen, T., Werringloer, J., & Marquardt, P. (1967) Aflatoxin B_1 and histamine in wine. *Arzneim. Forsch.* **17**, 888–890

(82) Stack, M. E., & Pohland, A. E. (1975) Collaborative study of a method for chemical confirmation of the identity of aflatoxin. *JAOAC* **58**, 110–113

(83) Crisan, E. V., & Grefig, A. T. (1968) The formation of aflatoxin derivatives. *Contrib. Boyce Thompson Inst.* **24**, 3–8

(84) Marsh, P. B., Simpson, M. E., Ferretti, R. J., Campbell, T. C., & Donoso, J. (1969) Relation of aflatoxin in cotton seeds at harvest to fluorescence in the fiber. *J. Agric. Food Chem.* **17**, 462–467

(85) Marsh, P. B., Simpson, M. E., Ferretti, R. J., Merola, G. V., Donoso, J., Craig, G. O., Trucksess, M. W., & Work, P. S. (1969) Mechanism of formation of a fluorescence in cotton fibers associated with aflatoxins in the seeds at harvest. *J. Agric. Food Chem.* **17**, 468–472

(86) Marsh, P. B., Simpson, M. E., Craig, G. O., Donoso, J., & Ramey, H. H. (1973) Occurrence of aflatoxins in cotton seeds at harvest in relation to location of growth and field temperatures. *J. Environ. Qual.* **2**, 276–281

(87) Whitten, M. E. (1969) Screening cottonseed for aflatoxins. *J. Am. Oil Chem. Soc.* **46**, 39–40

(88) Cucullu, A. F., Pons, W. A., Jr., & Goldblatt, L. A. (1972) Fast screening method for detection of aflatoxin contamination in cottonseed products. *JAOAC* **55**, 1114–1119

(89) Velasco, J., & Whitten, M. E. (1973) Evaluation of Florisil tubes in detection of aflatoxin. *J. Am. Oil Chem. Soc.* **50**, 120–121

(90) Pons, W. A., Jr., Cucullu, A. F., Franz, A. O., Jr., Lee, L. S., & Goldblatt, L. A (1973) Rapid detection of aflatoxin contamination in agricultural products. *JAOAC* **56**, 803–807

(91) Pons, W. A., Jr., Cucullu, A. F., Franz, A. G., & Goldblatt, L. A. (1968) Improved objective fluorodensitometric determination of aflatoxins in cottonseed products. *J. Am. Oil Chem. Soc.* **45**, 694–699

(92) Pons, W. A., Jr. (1969) Collaborative study on the determination of aflatoxins in cottonseed products. *JAOAC* **52**, 61–72

(93) Pons, W. A., Jr., Cucullu, A. F., & Franz, A. O., Jr. (1972) Rapid quantitative TLC method for determining aflatoxins in cottonseed products. *JAOAC* **55**, 768–774

(94) Pons, W. A., Jr. (1975) Collaborative study of a rapid method for determining aflatoxins in cottonseed products. *JAOAC* **58**, 746–753

(95) Baur, F. J., & Armstrong, J. C. (1971) Collaborative study of a modified method for the determination of aflatoxins in copra, copra meal, and coconut. *JAOAC* **54**, 874–878

(96) *IUPAC Inf. Bull. Tech. Rep.* No. 9 (Aug. 1974)

(97) Shotwell, O. L., Goulden, M. L., & Hesseltine, C. W. (1972) Aflatoxin contamination: Association with foreign material and characteristic fluorescence in damaged corn kernels. *Cereal Chem.* **49**, 458–464

(98) Bothast, R. J., & Hesseltine, C. W. (1975) Bright greenish-yellow fluorescence and aflatoxin in agricultural commodities. *Appl. Microbiol.* **30**, 337–338

(99) Dantzman, J., & Stoloff, L. (1972) Screening method for aflatoxin in corn and various corn products. *JAOAC* **55**, 139–141

(100) Shotwell, O. L., & Stubblefield, R. D. (1972) Collaborative study of the determination of aflatoxin in corn and soybeans. *JAOAC* **55**, 781–788

(101) Shannon, G. M., Stubblefield, R. D., & Shotwell, O. L. (1973) Modified rapid screening method for aflatoxin in corn. *JAOAC* **56**, 1024–1025

(102) Barabolak, R., Colburn, C. R., & Smith, R. J. (1974) Rapid screening method for examining corn and corn-derived products for possible aflatoxin contamination. *JAOAC* **57**, 764–766

(103) Holaday, C. E., & Lansden, J. (1975) Rapid screening method for aflatoxin in a number of products. *J. Agric. Food Chem.* **23**, 1134–1136

(104) DiProssimo, V. (1974) Collaborative study comparing two methods for the determination of aflatoxins in pistachio nuts. *JAOAC* **57**, 1114–1120

(105) Scott, P. M. (1969) Analysis of cocoa beans for aflatoxins. *JAOAC* **52**, 72–74

(106) Scott, P. M., & Przybylski, W. (1971) Collaborative study of a method for the analysis of cocoa beans for aflatoxins. *JAOAC* **54**, 540–544

(107) *IUPAC Inf. Bull. Tech. Rep.* No. 8 (Aug. 1973)

(108) Levi, C. P., & Borker, E. (1968) Survey of green coffee for potential aflatoxin contamination. *JAOAC* **51**, 600–602

(109) Levi, C. P. (1969) Collaborative study on a method for detection of aflatoxin B_1 in green coffee beans. *JAOAC* **52**, 1300–1303

(110) Scott, P. M. (1968) Note on analysis of aflatoxins in green coffee. *JAOAC* **51**, 609

(111) Romer, T. R. (1973) Determination of aflatoxins in mixed feeds. *JAOAC* **56**, 1111–1114

(112) Stubblefield, R. D., Shannon, G. M., & Shotwell, O. L. (1973) Aflatoxin M_1 in milk: Evaluation of methods. *JAOAC* **56**, 1106–1110

(113) Purchase, I. F. H., & Steyn, M. (1967) Estimation of aflatoxin M in milk. *JAOAC* **50**, 363–366

(114) Jacobson, W. C., Harmeyer, W. C., & Wiseman, H. G. (1971) Determination of aflatoxins B_1 and M_1 in milk. *J. Dairy Sci.* **54**, 21–24

(115) *IUPAC Inf. Bull. Tech. Rep.* No. 11 (Aug. 1974)

(116) Pons, W. A., Jr., Cucullu, A. F., & Lee, L. S. (1973) Method for the determination of aflatoxin M_1 in fluid milk and milk products. *JAOAC* **56**, 1431–1436

(117) Stubblefield, R. D., & Shannon, G. M. (1974) Aflatoxin M_1: Analysis in dairy products and distribution in dairy foods made from artificially contaminated milk. *JAOAC* **57**, 847–851

(118) Stubblefield, R. D., & Shannon, G. M. (1974) Collaborative study of methods for the determination and chemical confirmation of aflatoxin M_1 in dairy products. *JAOAC* **57**, 852–857

(119) Schuller, P. L., Verhülsdonk, C. A. H., & Paulsch, W. E. (1973) Analysis of aflatoxin M_1 in liquid and powdered milk. *Pure Appl. Chem.* **35**, 291–296

(120) Rossi, C. M., Borgatti, A. R., Cortesi, P., & Crisetig, G. (1971) Determination of aflatoxins in biological materials. *Atti Soc. Ital. Sci. Vet.* **25**, 437–441; thru (1972) *Chem. Abstr.* **77**, 84077

(121) Brown, N. L., Nesheim, S., Stack, M. E., & Ware, G. M. (1973) Method for the determination of aflatoxin in animal tissue. *JAOAC* **56**, 1437–1439

(122) Stack, M. E., Pohland, A. E., Dantzman, J. G., & Nesheim, S. (1972) Derivative method for chemical confirmation of identity of aflatoxin M_1. *JAOAC* **55**, 313–314

(123) Trucksess, M. (1976) Derivatization procedure for identification of aflatoxin M on a thin layer chromatogram. *JAOAC* **59**, 722–723

(124) Rodricks, J. V. (1973) Criteria for mycotoxin standards. *JAOAC* **56**, 1290–1291

(125) Rodricks, J. V., Stoloff, L., Pons, W. A., Jr., Robertson, J., & Goldblatt, L. A. (1970) Molar absorptivity values for aflatoxins and justification for their use as criteria of purity and analytical standards. *JAOAC* **53**, 96–101

(126) Rodricks, J. V., & Stoloff, L. (1970) Determination of concentration and purity of aflatoxin standards. *JAOAC* **53**, 92–95

(127) Purchase, I. F. H., & Altenkirk, B. (1973) Collaborative study of the stability of aflatoxin M_1 standards. *JAOAC* **56**, 1115–1118

(128) *IUPAC Inf. Bull. Tech. Rep.* No. 6 (Nov. 1972)

(129) Rodricks, J. V., & Stoloff, L. (1971) Collaborative study of a method for determination of concentration and purity of aflatoxin standards and use of the method for measuring stability of standards. *IUPAC Inf. Bull. Tech. Rep.* No. 1, 18 pp.

DISINFECTANTS

Analysis and Optimization of a Quantitative Organic Soil Neutralization Test for Disinfectants

ELAINE J. WHITMORE and NORMAN A. MINER
Arbrook, Inc., Arlington, TX 76010

An analysis and optimization of a test to quantitatively assess the resistance of disinfectants to neutralization by organic soil is presented. The recommended method uses sterile, dry baker's yeast as a standard organic soil, and 24-hr cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa* as test organisms. The test determines the maximum per cent (w/v) of organic soil which a disinfectant can tolerate and remain able to kill about 10^6 test organisms/ml in 10 min at 25°C. This per cent is defined as the organic soil neutralization number. The procedure offers several advantages over other organic soil capacity tests.

Disinfectants can be deactivated as a result of interaction with organic soil such as blood, mucus, and other organic matter (1, 2). Neutralization of disinfectants by organic soil may contribute to instances of disinfectant failures as well as to actual contamination of disinfectants with microorganisms, as have often been reported (3–8).

In the United States, disinfectants are regulated by the Environmental Protection Agency (EPA). The basic test required to assess disinfectant activity is the AOAC use-dilution method (9). This test determines the ability of a disinfectant, diluted with sterile distilled water to the recommended use-dilution, to disinfect stainless steel cylinders contaminated with test organisms (*Salmonella choleraesuis, Staphylococcus aureus,* and *Pseudomonas aeruginosa*) in 10 min at 20°C. There is no extraneous organic soil in this test and there are no standard tests required by the EPA to determine the activity of disinfectants in the presence of organic soil. Thus, chemicals which qualify as disinfectants at dilute concentrations in the laboratory may fail when they encounter organic soil in the reality of the use situation in the hospital, where disinfectants may be used repeatedly, or over a protracted period of time.

A reproducible quantitative test is presented which determines the per cent (w/v) of sterile, dry baker's yeast which a disinfectant at the manufacturer's recommended concentration (use-dilution) can tolerate and maintain the ability to kill about 10^6 bacteria/ml in 10 min at 25°C. The maximum per cent of organic soil (sterile, dry baker's yeast) in which a disinfectant remains active is defined as the organic soil neutralization number. An earlier publication (10) briefly described a preliminary version of this test as used in a study to comparatively evaluate several disinfectants.

This paper presents an analysis of the component factors of the test and establishes a standard set of experimental constants for determining the resistance of disinfectants to neutralization by organic soil.

Experimental

Reagents

(a) *Baker's yeast.*—Fleischmann's active dry yeast (manufactured by Standard Brands, Inc., New York, NY).

(b) *Disinfectants.*—Major active ingredients as listed on labels: aldhehydes—1, 2% acidic glutaraldehyde, pH 2.7–3.7; 2, 2% alkaline glutaraldehyde, pH 8.2–8.9. Phenolics—1, *o*-phenylphenol, 5%; *o*-benzyl-*p*-chlorophenol, 4.5%; *p-tert*-amylphenol, 1%; 2, *o*-phenylphenol, 10%; *o*-benzyl-*p*-chlorophenol, 8.5%; *p-tert*-amylphenol, 2%; 3, *o*-phenylphenol, 5.7%; *p-tert*-amylphenol, 1.8%. Cationic quaternary ammonium compounds (quats)—1, alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride, 9%; 2, alkyl dimethyl benzyl ammonium chloride, 3.25%; 3, *n*-alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride, 3.9%.

(c) *Subculture media.*—*See* 4.001(a), (c) (9). *Nutrient broth.*—Use with aldehyde disinfectants. *Letheen broth.*—Use with phenolic and quaternary ammonium disinfectants.

The effectiveness of any subculture medium in neutralizing carried-over disinfectant must be established for other categories of disinfectants before evaluating this test. To do this, add one loop-

Table 1. Determination of neutralization number

Test bacteria	Growth of Test Bacteria in Subculture Tubes g Yeast/10 ml disinfectant										Neut. No.
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	
S. aureus	0	0	0	0	0	0	0	0	0	+	
	0	0	0	0	0	0	0	0	0	+	9
P. aeruginosa	0	0	0	0	0	+	+	+	+	+	
	0	0	0	0	0	0	+	+	+	+	5

ful of disinfectant at manufacturer's recommended concentration to duplicate tubes containing 10 ml subculture medium. Inoculate separate tubes with 50–100 organisms from 24-hr cultures of each test bacteria and incubate tubes 48 hr at 37°C. If test bacteria grow in subculture medium, carried-over disinfectant has been neutralized and/or diluted sufficiently to eliminate bacteriostatic activity.

(d) *Test organisms.*—*S. aureus,* ATCC 6538; and *P. aeruginosa,* ATCC 15442; *see* 4.010–4.011 (9).

Concentration range of 24-hr cultures of test organisms used in these tests was $2.7–7.0 \times 10^8$/ml for *S. aureus* and $0.8–1.9 \times 10^9$/ml for *P. aeruginosa.* If necessary, dilute organisms with sterile 0.85% NaCl solution to achieve this concentration. Test bacteria should have resistance to phenol at 20°C as specified by 4.001–4.006 (9).

Preparation of Samples

(a) *Organic soil.*—Prepare yeast for tests by grinding to even consistency with mortar and pestle. For example, 20 g yeast ground for 60 sec in 300 ml mortar is suitable for use. Weigh dry yeast into 25×150 mm test tubes in desired increments to cover range of organic soil challenge to be tested (*see* example presented in Table 1). Cap tubes and sterilize yeast by autoclaving. Break up clumps of autoclaved yeast in test tubes by agitation on vortex-action mixer.

(b) *Disinfectants.*—Dilute each disinfectant to be tested to manufacturer's recommended concentration (use-dilution) in sterile distilled water.

Test Procedure

Aseptically add 10 ml disinfectant to each tube of dry yeast and mix thoroughly. Place tubes of disinfectant plus yeast in 25°C water bath for 1 hr.

Using 1 ml pipet, add 0.1 ml 24-hr culture of test organisms ($2.5–7.0 \times 10^8$ *S. aureus*/ml or $0.8–1.9 \times 10^9$ *P. aeruginosa*/ml) to each disinfectant-yeast tube at 30-sec intervals, mixing immediately prior to and following addition of organisms. Incubate tubes 10 min at 25°C. At 30-sec intervals, mix each tube, and using 4 mm id loop, transfer one loopful of yeast-disinfectant-organism mixture from each tube to 20×150 mm subculture tube containing 10 ml subculture medium. Agitate subculture tubes and transfer one loopful from each inoculated tube to second subculture tube containing 10 ml subculture medium. Agitate second set of tubes. Double subculture technique serves as additional check for bacteriostasis. If growth appears in second subculture tube while first subculture tube is negative, subculture medium has not adequately neutralized disinfectant carried over, and another neutralizing medium must be selected.

Incubate all subculture tubes 48 hr at 37°C and read results by macroscopic examination for turbidity as indicator of growth of test organism. At higher yeast concentrations, clarity of nutrient medium may be affected by yeast carry-over. Streak samples from such tubes onto letheen agar plates for verification of bacterial growth.

Calculation of Neutralization Number

Neutralization number is defined as maximum per cent (w/v) of yeast which will not inactivate disinfectant within parameters of this test. Obtain number by multiplying greatest weight of yeast resulting in no growth in first row of subculture tubes by 10. Because of limit of physical compatibility of disinfectants with yeast, 3 g yeast/10 ml disinfectant was maximum used. Thus, the highest measurable neutralization number was ≥ 30. An example is shown in Table 1.

Establishment of Test Parameters

The influences of several components of this test were evaluated in order to develop and recommend an optimum practical standard procedure which would assure accuracy and repeatability. The test components studied were: use of hard water vs. distilled water; length of disinfectant-organic soil contact time; number of test bacteria; age of test bacteria; incubation temperature; and disinfectant concentration.

Table 2. Neutralization numbers of disinfectants at their manufacturer's recommended use-dilutions

Disinfectant	Use-diln	% Active ingredients at use-diln	Neutralization No.			
			Distd water[a]		Hard water[b]	
			S. aureus	*P. aeruginosa*	*S. aureus*	*P. aeruginosa*
Aldehyde 1	undild	2.0	≥30	≥30	NT[c]	NT
Aldehyde 2	undild	2.0	≥30	≥30	NT	NT
Phenolic 1	1:33	0.315	4	6	4	3
Phenolic 2	1:256	0.080	1	0.5	1	<0.25
Phenolic 3	1:100	0.075	1	2	1	<0.25
Quat 1	1:128	0.070	2	1	2	0.5
Quat 2	1:32	0.102	1	0.5	1	<0.25
Quat 3	1:32	0.122	3	3	3	1

[a] Dilutions of phenolic and quat disinfectants were made with sterile distilled water.
[b] Dilutions of phenolic and quat disinfectants were made with sterile, 600 ppm standard hard water.
[c] Not tested; used undiluted.

Results of these studies, which are presented below, led to the selection of the set of test constants described in the *Test Procedure* section of this paper. Examples of application of the optimum test method to determine organic soil neutralization numbers of several disinfectants and examples of test reproducibility are also presented.

Results

Effect of Hard Water on Neutralization Numbers

The activity of some disinfectants is reduced in the presence of hard water (11). Table 2 indicates the effect on soil neutralization number of dilution of disinfectants in water of 600 ppm hardness or in distilled water (*see* **4.027–4.028**) (9). The activity against *P. aeruginosa* of all the phenolic and quat disinfectants tested was 50 to ≥75% less in hard water than in distilled water, as reflected in reduced neutralization numbers. When *S. aureus* was the test organism, neutralization numbers were not altered by dilution of the disinfectants in hard water. As a result of these different effects of hard water, sterile distilled water is used to dilute disinfectants in this organic soil neutralization test. This allows for standardization of the test, and avoids the necessity of making a judgment as to what is a representative water hardness. The effect of hard water on glutaraldehyde at the manufacturers' recommended concentration was not studied because both glutaraldehyde disinfectants are used undiluted.

Table 3. Effect of contact time between disinfectants and organic soil on neutralization numbers

Disinfectant	% Active ingredients at diln tested	Contact time, min	Neutralization No.	
			S. aureus	*P. aeruginosa*
Phenolic 1	0.25	0.5	10	>10
		2	7	4
		10	5	5
		30	5	5
Quat 3	0.25	0.5	>10	9
		2	7	6
		10	8	7
		30	7	7
Aldehyde 2	0.25	0.5	≥30	≥30
		2	30	22
		10	26	18
		30	14	10
		40	12	10
		60	12	10
Aldehyde 2	2.00	10	≥30	≥30
		30	≥30	≥30
		60	≥30	≥30
		300	≥30	≥30

Length of Disinfectant-Organic Soil Contact

The effect of the length of contact between the organic soil and disinfectant was determined in order to establish the appropriate time to use in the standard test procedure. Disinfectant was mixed with the yeast and allowed to remain in contact with the yeast from 30 sec to 5 hr. As shown in Table 3, neutralization of the phenolic and quat tested occurred within 10 min after the initial contact with the yeast, but was not complete in less than 2 min. Neutralization of 0.25% alkaline glutaraldehyde, however, was not complete until 30–40 min after initial contact with the yeast. The neutralization number of undiluted alkaline glutaraldehyde is apparently

Table 4. Neutralization numbers of disinfectants challenged with varying numbers of *P. aeruginosa*

Disinfectant[a]	Final concn of *P. aeruginosa*/ml disinfectant			
	10^7	10^6	10^5	10^4
Phenolic 1	6	6	8	12
Phenolic 2	3	3	7	>10
Quat 1	3	5	9	>14
Quat 3	8	12	>18	>18

[a] Each disinfectant was tested at 0.25% active ingredients.

Table 5. Effect of age of test culture on neutralization numbers

Disinfectant[a]	Neutralization No.			
	S. aureus		*P. aeruginosa*	
	24 hr	48 hr	24 hr	48 hr
Phenolic 1	3	4	6	5
Quat 3	8	7	8	8
Aldehyde 2	10	10	8	10

[a] Each disinfectant was tested at 0.25% active ingredients.

sufficiently in excess of 30 (the highest number possible in this test) that no change in neutralization number was observed within a 5-hr disinfectant-yeast contact time. Because the same degree of neutralization of alkaline glutaraldehyde was achieved in 1 hr as in 5 hr, and because 1 hr is a more practical time than 5 hr, a standard contact time of 1 hr is recommended to allow maximum neutralization of all disinfectants prior to adding the test microorganisms.

Effect of Numbers of Organisms

The ability of any disinfectant to completely kill microorganisms in a fixed exposure time is a function of the number of microorganisms with which the disinfectant is challenged. Neutralization numbers for several disinfectants were determined by using various concentrations of *P. aeruginosa* in the inoculum as the only test variable. The test cultures were diluted in sterile 0.85% sodium chloride solution. As expected, the neutralization numbers decreased as the number of organisms/ml in the disinfectant was increased from 10^4 to 10^7 (Table 4). The smallest differences in neutralization numbers occurred when the concentration of *P. aeruginosa* was in the range of 10^6 to 10^7 *P. aeruginosa*/ml disinfectant. The results indicated that the standard test procedure should use 10^6 to 10^7 microorganisms/ml disinfectant to achieve consistent neutralization numbers.

Effect of Age of Culture

Virtually no differences in neutralization numbers were noted when 48-hr cultures of *S. aureus* or *P. aeruginosa* were used rather than 24-hr cultures (Table 5). For convenience, 24-hr cultures that achieve 2.5–7 $\times$ 10^8 *S. aureus*/ml culture and 0.8–1.9 $\times$ 10^9 *P. aeruginosa*/ml culture are the standard test organisms for the organic soil neutralization test.

Effect of Temperature

As shown in Table 6, minimal differences in neutralization numbers were observed when the disinfectants were tested at 20 or 25°C. Neutralization numbers were the same or slightly higher at 25 than at 20°C. Since water baths able to achieve 25°C are more readily available than refrigerated baths, 25°C was determined as the standard temperature for this test.

Effect of Disinfectant Concentration

To assess the effect of disinfectant concentration on resistance to inactivation by organic soil, neutralization numbers were determined for several disinfectants diluted with sterile distilled water to provide various levels of active ingredients. Concentration-response curves of representative disinfectants are presented in Figs. 1–5. As indicated in these figures, there was a linear increase in neutralization number with an increase in the concentration of active ingredients. "Active ingredients" refers only to the ingredients in the major chemical group of each disinfectant, i.e., phenolics, quats, or glutaraldehyde.

Table 6. Effect of temperature on neutralization numbers

Disinfectant[a]	Neutralization No.			
	S. aureus		*P. aeruginosa*	
	20°C	25°C	20°C	25°C
Phenolic 1	4	4	5	5
Quat 3	7	8	8	8
Aldehyde 2	12	14	10	12

[a] Each disinfectant was tested at 0.25% active ingredients.

This paper was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

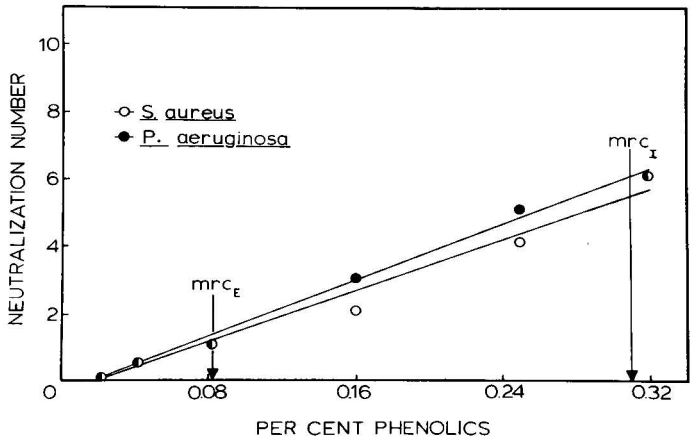


FIG. 1—Effect of concentration of phenolic disinfectant 1 on organic soil neutralization number; mrc$_E$ = manufacturer's recommended concentration (use-dilution) for environmental disinfection; mrc$_I$ = manufacturer's recommended concentration for instrument disinfection.

Neutralization Numbers of Disinfectants at Manufacturer's Recommended Use-Dilutions

The neutralization numbers of each test disinfectant at the manufacturer's recommended use-dilution against *S. aureus* and *P. aeruginosa* as determined by the standard test method are shown in Table 2. Alkaline and acidic glutaraldehyde (2%) were able to tolerate at least 30% (w/v) organic soil in the form of sterile, dry baker's yeast without losing the ability to kill the test organisms in 10 min (neutralization numbers of $\geqslant$30). The neutralization numbers of the quats tested ranged from 0.5 to 3.0 for *P. aeruginosa* and from 1.0 to 3.0 for *S. aureus*, while the neutralization numbers of the phenolics tested ranged from 0.5 to 6.0 for *P. aeruginosa* and from 1.0 to 4.0 for *S. aureus*.

Test Reproducibility

Organic soil neutralization tests were repeated 5 times per day for 5 different days for 4 disinfectants, using *P. aeruginosa* as the test organism. The disinfectants were diluted in sterile distilled water to the level of 0.25% active in-

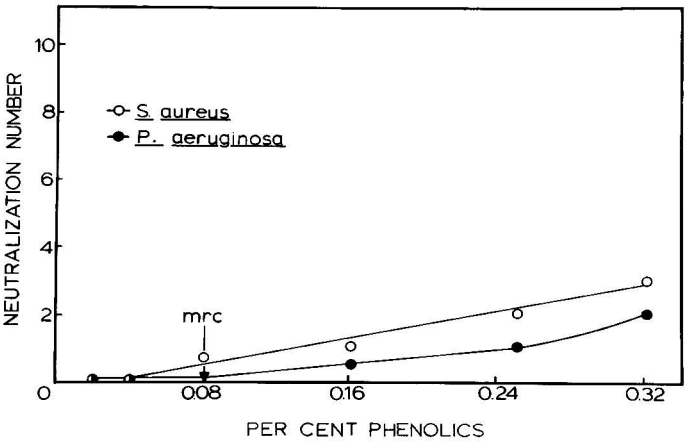


FIG. 2—Effect of concentration of phenolic disinfectant 2 on organic soil neutralization number; mrc = manufacturer's recommended concentration (use-dilution).

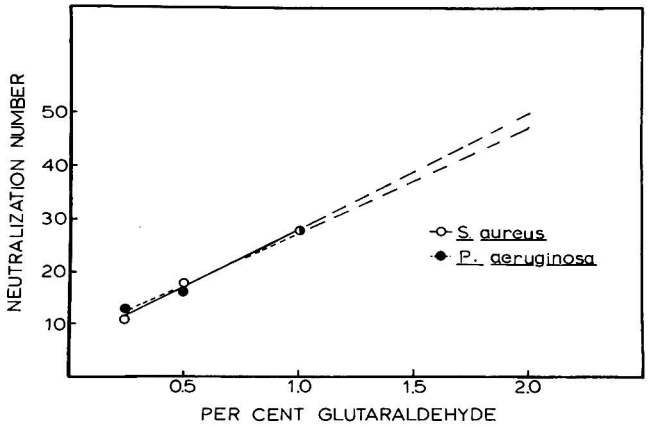


FIG. 3—Effect of concentration of aldehyde disinfectant 2 on organic soil neutralization number; manufacturer's recommended concentration (use-dilution) = 2.0%.

gredients (i.e., glutaraldehyde, total phenolics, total quats). As shown in Table 7, the standard deviations of each group of 25 samples ranged from 6.5 to 12.6% of the means. In these tests, yeast increments of 0.1 g were used for all disinfectants except aldehyde 2, for which 0.2 g increments were used. Deviation can be minimized by reducing the size of organic soil increments in the test. For example, a test using 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 g yeast (0.1 g increments) will yield statistically better results than a test using 0.2, 0.4, and 0.6 g yeast (0.2 g increments).

Not all disinfectants were tested in all segments of the research described. Representative disinfectants were evaluated, and the overall influence of each test component was similar for each.

Discussion

The organic soil neutralization test offers several advantages over British organic soil capacity tests such as B.S. 808:1938 (12) and the Kelsey-Sykes capacity test (13). The organic soil neutralization test presented here is a quantitative test, providing a numerical indication of

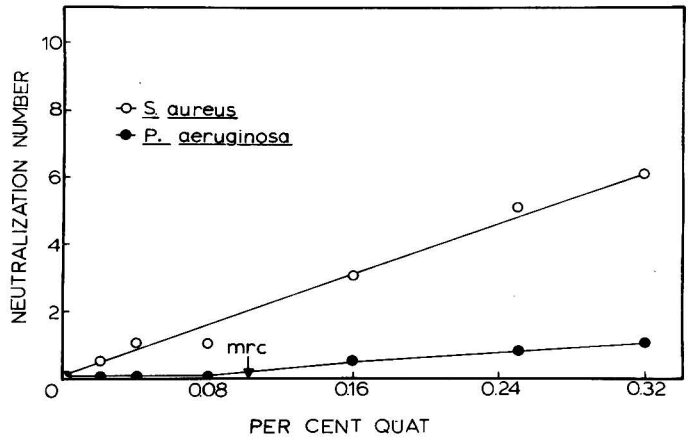


FIG. 4—Effect of concentration of quat disinfectant 2 on organic soil neutralization number; mrc = manufacturer's recommended concentration (use-dilution).

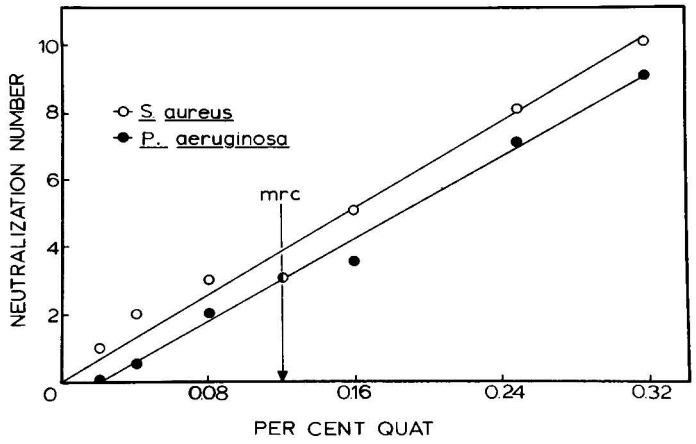


FIG. 5—Effect of concentration of quat disinfectant 3 on organic soil neutralization number; mrc = manufacturer's recommended concentration (use-dilution).

the resistance to neutralization based on test procedures carefully controlled with regard to inanimate factors and microorganisms (i.e., number, age, resistance to phenol). Unlike the latter 2 methods, no subjective judgment concerning a maximum percentage of organic soil representative of a use situation needs to be made. In addition, the British tests add the organic soil and test bacteria simultaneously, and timing begins with that addition. This procedure may lead to inaccurate conclusions because neutralization of disinfectants is not instantaneous, but requires a finite time dependent on the type and concentration of the disinfectant. By not allow-

Table 7. Reproducibility of organic soil neutralization test

Disinfectant[a]	Day	Neutralization No. (*P. aeruginosa*)	Mean	Std dev.[b]
Aldehyde 2	1	12, 12, 12, 14, 14	12.8	0.98
	2	14, 14, 14, 12, 12	13.2	0.98
	3	12, 12, 12, 12, 12	12.0	0
	4	12, 12, 12, 12, 10	11.6	0.80
	5	14, 14, 14, 14, 14	14.0	0
		all numbers	12.72	1.11
Phenolic 1	1	6, 6, 5, 5, 5	5.4	0.49
	2	5, 5, 4, 4, 4	4.4	0.49
	3	4, 4, 4, 5, 5	4.4	0.49
	4	5, 5, 5, 5, 6	5.2	0.40
	5	4, 5, 5, 5, 5	4.8	0.40
		all numbers	4.84	0.61
Phenolic 2	1	3, 3, 3, 3, 3	3.0	0
	2	3, 3, 3, 3, 3	3.0	0
	3	3, 3, 3, 3, 4	3.2	0.4
	4	3, 3, 3, 3, 3	3.0	0
	5	3, 3, 3, 3, 3	3.0	0
		all numbers	3.04	0.20
Quat 3	1	9, 8, 8, 9, 9	8.6	0.49
	2	8, 7, 8, 8, 8	7.8	0.40
	3	7, 7, 7, 8, 8	7.4	0.49
	4	8, 8, 8, 7, 7	7.6	0.49
	5	8, 8, 8, 8, 8	8.0	0
		all numbers	7.88	0.59

[a] All disinfectants were tested at 0.25% active ingredients.

[b] Standard deviation = $\sqrt{[\Sigma (x-\bar{x})^2]/N}$.

ing an adequate contact period between the disinfectant and the soil, maximum inactivation of the disinfectant will not be realized when the test organisms are introduced, which could result in erroneously high indications of the resistance of the disinfectant to neutralization by organic soil. Under use conditions, a disinfectant which is used repeatedly or over a protracted period of time will remain in contact with organic soil for prolonged periods and will undergo maximum neutralization. The organic soil neutralization test described in this paper provides for maximum inactivation of the disinfectant by allowing a contact period of 1 hr between the disinfectant and yeast prior to the addition of the test bacteria.

A test which quantitatively evaluates the ability of a disinfectant to remain active in the presence of organic soil is needed. Disinfection failures, as well as growth of bacteria in disinfectant solutions, can occur as a result of ignorance of the inactivating effect of organic soils on disinfectants. Such disinfectant failures in hospitals are often associated with outbreaks of infection (3, 6).

The purpose of this organic soil neutralization test is to provide a number indicative of the relative and absolute ability of a disinfectant to resist neutralization by organic soil. If the organic soil neutralization numbers for *S. aureus* and *P. aeruginosa* were determined for and displayed by all disinfectants, consumer evaluation of the potential activity of each disinfectant in *specific* use situations would be facilitated, and the incidence of disinfectant failures could be expected to be reduced. It is therefore suggested that a test such as presented here be considered for incorporation into AOAC disinfectant testing and evaluation methods.

Received August 15, 1975. Resubmitted April 5, 1976.

Acknowledgments

The authors wish to thank M. L. McBee, R. F. Berry, and A. K. Ayers for their expert technical assistance, and S. Klasky and J. W. McDowell for their valuable and helpful critical review of this manuscript.

REFERENCES

(1) Hedgecock, L. W. (1967) *Antimicrobial Agents,* Lea & Febiger, Philadelphia, PA, p. 18
(2) Sykes, G. (1967) *Disinfection and Sterilization,* J. B. Lippincott Co., Philadelphia, PA, pp. 24–25
(3) Lee, J. C. (1961) *J. Am. Med. Assoc.* **177,** 144–146
(4) Simmons, N. A., & Gardner, D. A. (1969) *Brit. Med. J.* **2,** 668–669
(5) *Ann. Intern. Med.* (1970) **72,** 282–283
(6) Center for Disease Control (1974) *National Nosocomial Infections Study,* 4th Quarter 1973, 18–23, Atlanta, GA
(7) Burdon, D. W., & Whitby, J. L. (1967) *Brit. Med. J.* **2,** 153–155
(8) Litsky, B. Y., & Litsky, W. (1968) *Am. J. Public Health* **58,** 534–543
(9) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC
(10) Miner, N. A., Whitmore, E., & McBee, M. L. (1975) *Dev. Indust. Microbiol.* **16,** 23–30
(11) Winicov, M., Schmidt, W., & Palochak, M. (1974) *JAOAC* **57,** 880–884
(12) *British Standard Specifications* (1960) B.S. 808:1938, Modified Technique of the Chick-Martin Test for Disinfectants, British Standards Institution, London, England
(13) Kelsey, J. C., & Sykes, G. (1969) *Pharm. J.* **202,** 607–609

FORENSIC SCIENCES

Identification of Human Seminal Acid Phosphatase by Electrophoresis

MARK D. STOLOROW, JAMES D. HAUNCHER, and WILLARD C. STUVER*
Michigan State Police Scientific Laboratory, 42145 W Seven Mile Rd, Northville, MI 48167
** Pittsburgh and Allegheny County Crime Laboratory, 311 Ross St, Pittsburgh, PA 15219*

Recent advances in forensic science in the identification of human seminal acid phosphatase are presented, with particular attention to the acrylamide gel electrophoretic method. In that method a difficulty in distinguishing seminal acid phosphatase from certain fecal phosphatases has been observed and an attempt is made here to distinguish the phosphatases from one another experimentally by differential substrate specificity. 4-Methylumbelliferyl phosphate and α-naphthyl acid phosphate are used as reaction substrates. Although seminal and vaginal acid phosphatases are differentiated by the modified method, fecal phosphatase is not clearly differentiated from seminal acid phosphatase.

For years forensic scientists have sought a reliable method for confirming human seminal stains in cases where spermatozoa were absent. A stable constituent of seminal plasma was sought which would meet 3 criteria in the analysis of seminal stains: persistence, abundance, and uniqueness.

Of all the components of seminal plasma for which forensic scientists have developed testing procedures, perhaps none has more closely approached fulfilling these criteria or received as much attention as the enzyme acid phosphatase. Acid phosphatase demonstrates both excellent persistence and abundance in seminal stains (1). However, acid phosphatase is not unique to semen but is also present in varying concentrations in other body fluids, including vaginal secretions and saliva. This limits its usefulness as a test of uniqueness for seminal acid phosphatase in aspermatic stains composed of semen, vaginal secretions, or saliva, or mixtures of these secretions.

In our experience, merely measuring the enzymatic activity of acid phosphatase in mixed stains may be inadequate to confirm the acid phosphatase as distinctly seminal in origin, particularly in forensic cases involving minute or weak stains.

L-Tartrate is a well known inhibitor of seminal acid phosphatase. However, evidence that L-tartrate inhibition is not specific for seminal acid phosphatase is well documented (2–5). A number of additional methods have been devised to characterize the uniqueness of this acid phosphatase.

In 1973, Baxter (2) reported the identification of human seminal acid phosphatase by electro - immunodiffusion and immunoelectrophoretic techniques which employ a precipitating antiserum and the substrate α-naphthyl phosphoric acid. Baxter's techniques depend on both the immunological character and the electrophoretic mobility of human seminal acid phosphatase to differentiate this enzyme from the acid phosphatases in other body secretions.

He found that testing seminal acid phosphatase *only* for its immunological activity by the Ouchterlony double diffusion technique (2) fails to differentiate semen from vaginal fluid. Baxter noted that "specific" antihuman-semen serum is species-specific but, unfortunately, cross-reacts with human vaginal fluid when stained with α-naphthyl phosphoric acid and Brentamine Fast Dark Blue R, and with vaginal fluid, saliva, and tears when stained with amido black.

Consequently, Baxter turned to an immunoelectrophoretic technique on agar, combining the electrophoretic mobility of acid phosphatases with their antigenic properties to identify the presence of human semen.

Several months before Baxter published his findings, Lam *et al.* (3) reported separating acid phosphatases found in body tissues by acryl-

J. D. Hauncher is Associate Referee on Biological Fluids (Immunoelectrophoresis).

This paper was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

amide gel electrophoresis into 5 component isoenzymes. They found that acid phosphatase extracted from pulverized prostatic tissue contains primarily 3 component isoenzymes which they termed isoenzymes 1, 2, and 4. Experiments with liquid semen revealed that the only isoenzyme of acid phosphatase present in semen is isoenzyme 2.

Based on these findings, Lam *et al.* concluded that the acid phosphatase isoenzymes 1 and 4 in prostatic tissue are intracellular in origin while isoenzyme 2 is an extracellular enzyme secreted by the prostate into seminal plasma.

Although Lam *et al.* were investigating acid phosphatase for purposes of medical research, it was of particular interest to forensic scientists that they were able to distinguish prostatic acid phosphatase from that of 19 other tissues *solely* on the basis of electrophoretic mobility in an acrylamide gel support medium.

To the frustration of forensic scientists, Lam and his colleagues included neither vaginal fluid nor saliva among the physiological specimens selected for investigation.

However, in 1974, Emes (neé Adams) and Wraxall (4) reported success with a method of acrylamide gel electrophoresis which differentiates seminal acid phosphatase from other acid phosphatases. They did investigate the electrophoretic mobilities of acid phosphatases from vaginal secretions and saliva and found their electrophoretic patterns readily distinguishable from that of seminal acid phosphatase. They also analyzed other sources of acid phosphatase, including animal semen, vegetable extracts, human blood, and human feces. Emes and Wraxall concluded that acid phosphatase from human semen could be differentiated from each of the other substances investigated.

In reviewing forensic science's history of attempts to devise methods to characterize the uniqueness of human seminal plasma, the work of Emes and Wraxall represents a milestone in at least 2 respects.

First, the method of Emes and Wraxall, like Baxter's method, differentiates human seminal acid phosphatase from other acid phosphatases. Second, the method of Emes and Wraxall provides a means of identifying human seminal acid phosphatase solely on the basis of electrophoretic mobility. Their technique does not require the use of antisera, in contrast with Baxter's method which is dependent on both the immunological and the electrophoretic properties of acid phosphatase.

The method of Emes and Wraxall has been used in casework in our laboratories on a regular basis over the past 24 months. In our experience, we consider the technique of considerable value in differentiating seminal from vaginal acid phosphatase. However, inadequate differentiation of semen from fecal matter on certain anal swabs and stained material using this technique has given us cause for concern and led us to the present investigation.

Emes and Wraxall described their method as one which will ". . . distinguish semen from faeces and vegetable material by electrophoretic mobility and differences in pH activity . . ." (4).

It is equally important to repeat their warning concerning phosphatases in feces: "Phosphatases in other body secretions and vegetable extracts are likely to be encountered in forensic work. Of these the most common is faeces and care must be exercised when interpreting the results" (4).

Emes and Wraxall observed that fecal phosphatase exhibits a considerably higher pH optimum (pH 11.0) than seminal acid phosphatase (which has a pH optimum of 5.5 to 7.0 (3)). Emes and Wraxall suggested that the use of a reaction buffer of pH 3.0 significantly diminishes or eliminates the bands of fecal phosphatase on development with the substrate 4-methylumbelliferyl phosphate (MUP). In addition they observed that the sites of fecal phosphatase activity ". . . varied from donor to donor and day to day, but did not correspond with the semen or vaginal acid phosphatase bands" (4). Accordingly, the presence of seminal acid phosphatase on anal swabs is presumably identifiable by its characteristically strong band intensity combined with its characteristic electrophoretic mobility.

However, in our laboratories control anal swabs free of semen have been analyzed by the method of Emes and Wraxall and it was observed that certain of these samples produced a single band of weak intensity with an electrophoretic mobility corresponding to seminal acid phosphatase (Fig. 1).

G. T. Duncan (1975, Broward County Crime Laboratory, Ft. Lauderdale, FL, private communication) reported that, on several occasions,

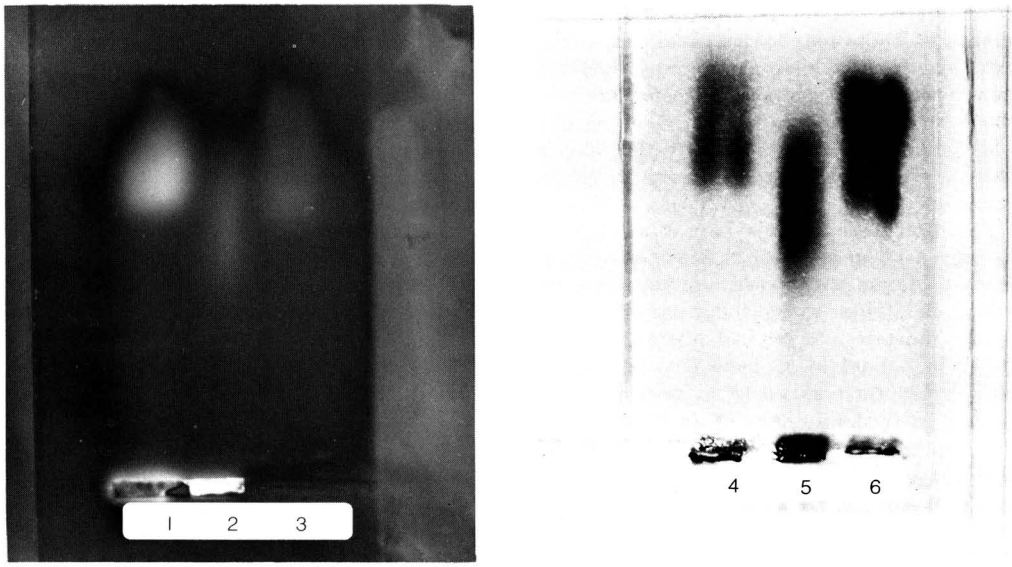


FIG. 1—Acrylamide gel electrophoresis plate bearing 6 control specimens of human secretion stains: 1 and 4, semen-free anal swab; 2 and 5, semen-free vaginal swab; 3 and 6, seminal stain. The left side of the plate was developed with MUP and the right side with sodium α-naphthyl acid phosphate and naphthanil diazo blue B. The anode corresponds to the top of the figure.

analysis of semen-free anal swabs by the method of Emes and Wraxall produced intense bands of activity at a position corresponding to seminal acid phosphatase. In other instances, Duncan observed an intense "fast" band from semen-free anal swabs at a position slightly anodic to seminal acid phosphatase.

Weak or dilute seminal stains are not uncommon evidence in criminal casework. Consequently, in cases where a minute quantity of semen is present in a stain or on a swab suspected of being contaminated with fecal matter, the forensic scientist might be less than confident in concluding that the faint electrophoretic band in the position of seminal acid phosphatase is in fact from semen and not from fecal matter.

In an attempt to resolve this problem, differences in substrate specificity between acid phosphatase and fecal phosphatases were considered.

In his recent study of acid phosphatases, Sensabaugh (5) separated human acid phosphatases into 4 major classes according to molecular weight. One of the variations he observed among these classes of acid phosphatase was a variation of enzymatic substrate specificity. It appears that the lower molecular weight classes of acid phosphatase (including erythrocyte acid phosphatase) react readily with MUP but not with α-naphthyl acid phosphate. Conversely, the higher molecular weight classes, which include seminal acid phosphatase, exhibit strong reactivity with α-naphthyl acid phosphate.

Based on Sensabaugh's observation that acid phosphatases vary in substrate specificity, it was decided to conduct electrophoresis by the method of Emes and Wraxall and compare the relative reactivities of MUP and α-naphthyl acid phosphate with fecal phosphatase.

METHOD

The 1 mm thick horizontal polyacrylamide gel electrophoretic method for identifying seminal acid phosphatase is based on that described by Emes and Wraxall (4). The few minor technical modifications are those employed in the State Police Scientific Laboratory, Northville, MI.

Apparatus

(a) *Electrophoretic apparatus.*—Horizontal electrophoresis tank (Shandon Southern Instruments, Inc., Sewickley, PA, No. SAE-3225, Kohn Model U-77); cooling plate (Shandon SAE-3231 TLE

platen and accessories); and power supply Vokam constant voltage-constant current-stabilized dc power supply (Shandon SAE-2761). Operating parameters: 135 v for 16 hr with cooling plate on for 18 hr in 4°C refrigerator; current should not exceed 20 ma at onset. Acid phosphatases migrate toward anode in this method.

(b) *Ultraviolet viewing box.*—Chromato-Vue Model CC-20 portable darkroom (Ultra-Violet Products, Inc., San Gabriel, CA).

(c) *Electrophoresis plate.*—1 mm deep. Glass plate, 8½ × 6″, bordered with glass or polycarbonate strips ¼″ wide × 1 mm thick.

Reagents

(a) *Tank buffer.*—Veronal buffer. Prepare 2.0 L aqueous solution containing 0.021*M* sodium barbitone (4.35 g/L) and 0.013*M* barbital (diethylbarbituric acid, 2.39 g/L). Adjust to pH 8.5 with 0.10*N* NaOH.

(b) *Gel buffer.*—Use undiluted tank buffer.

(c) *Reaction buffer.*—Prepare 0.5 L aqueous solution of 0.05*M* citric acid (10.5 g of monohydrate/L). Adjust with 0.10*N* NaOH to pH 3.0.

(d) *Acrylamide.*—Cyanogum 41 gelling agent (Fisher Scientific Co., No. C-588).

(e) *Polymerization activator.*—Ammonium persulfate (Fisher Scientific Co., Pittsburgh, PA, No. A-682).

(f) *Polymerization catalyst.*—*N,N,N′,N″*-tetramethylethylenediamine (TEMED) (Sigma Chemical Co., St. Louis, MO, No. T-8133).

(g) *Hydrolyzed starch.*—Electrophoresis grade. Connaught, hydrolyzed (Fisher Scientific Co., No. S-676).

(h) *Acid phosphatase reaction substrate.*—(*1*) 4-Methylumbelliferyl phosphate (MUP), sodium salt (ICN Pharmaceuticals, Inc., Cleveland, OH, No. 102359). (*2*) Sodium α-naphthyl acid phosphate (Dajac Laboratories, Philadelphia, PA, No. 554421).

(i) *Coupling dye.*—Naphthanil diazo blue B (Dajac Laboratories, No. 553322).

(j) *Agar.*—Oxoid purified agar (Colab Laboratories, Inc., Glenwood, IL).

Preparation of Polyacrylamide Gel

Prepare 8.0% polyacrylamide gel by weighing 5.0 g acrylamide and 0.07 g ammonium persulfate. Dissolve in 50 ml gel buffer and degas. Add 20 μl TEMED to gel solution to catalyze polymerization. Pour gel solution onto glass plate promptly. Lower second glass plate onto acrylamide solution, excluding air bubbles from solution. Polymerization is complete in 10–15 min at room temperature. Remove upper glass plate. Slice and remove strip of acrylamide 4 mm × 10 cm long from a position one-third the way from cathode end of gel.

Preparation of Starch Stacking Gel

Prepare 7.8% starch gel solution by mixing hydrolyzed starch with 25.0 ml gel buffer; boil and degas. Cool starch solution to 55°C and pipet into trough on plate. Place plate in moisture chamber and store 30 min at 4°C or until starch solution has gelled.

Preparation of Samples

Cut samples measuring 2 × 10 mm from stains on cloth or swabs. (Width may vary, depending on relative concentration of stain.) Include control stains of known semen and known vaginal secretion in each trial. (A single 1 cm cotton thread from a prepared seminal stain is ample for a seminal acid phosphatase control.) On microscope slides with well depressions, soak samples in minimum of distilled water and store 5–10 min in moisture chamber at room temperature. Exercising care to avoid cross contamination, insert samples into starch gel origin. Proceed with electrophoresis according to parameters stated under *Apparatus.*

Determination

(a) *MUP.*—Dissolve 4.0 mg MUP in 10.0 ml reaction buffer (pH 3.0). Saturate 5 × 5″ piece of Whatman 3MM chromatographic paper with reaction mixture and place on top of gel anodic to origin, taking care to exclude air bubbles. Store plate 10–20 min in moisture chamber at 37°C. Read results under longwave ultraviolet illumination.

(b) *α-Naphthyl acid phosphate.*—On surface of polyacrylamide gel, construct mold using 1 mm thick glass or polycarbonate strips to mark out area measuring 5 × 5″ and positioned anodic to origin. Prepare solution containing 5.0 mg sodium α-naphthyl acid phosphate, 5.0 mg naphthanil diazo blue B, and 10.0 ml reaction buffer (pH 3.0). Prepare second solution by adding 0.16 g agar to 10.0 ml reaction buffer (pH 3.0); boil and degas. Cool agar solution to 48–50°C. Mix solution containing α-naphthyl acid phosphate into agar solution and pour combined reaction mixture promptly into mold on top of polyacrylamide gel. Store plate 30–60 min in 37°C moisture chamber. Purple bands develop at sites of enzymatic activity.

Experimental

In order to demonstrate whether fecal phosphatase shows different sensitivity to the 2 substrates, MUP and α-naphthyl acid phosphate, the following experiment was performed.

On a polyacrylamide gel prepared according to

the method above, 6 samples were inserted into the starch gel origin. Inserted into sample slots 1 and 4 were 2 portions of a single control anal swab (semen-free). Inserted into sample slots 2 and 5 were 2 portions of a single control vaginal swab (semen-free). Inserted into sample slots 3 and 6 were 2 portions of a single control seminal stain.

Electrophoresis was carried out on a cooling plate for 16 hr at a constant voltage of 135 v, using a continuous Veronal tank buffer (pH 8.5) as described above.

One-half of the plate (Samples 1, 2, and 3) was developed with the MUP reaction mixture. The other half of the plate (Samples 4, 5, and 6) was developed with the reaction mixture containing α-naphthyl acid phosphate. Following development, each half of the plate was photographed, using black and white film. (Each half of the plate was intentionally developed for 2½ hr rather than the normal 30–60 min period to maximize the intensity of the bands for photographic purposes. Consequently, the bands of enzymatic activity are unnecessarily broad and diffuse.)

Results and Discussion

The photographs (Fig. 1) show that the electrophoretic mobility of the fecal phosphatase control (Samples 1 and 4) corresponds to the mobility of seminal acid phosphatase (Samples 3 and 6).

The fecal phosphatase band patterns of Samples 1 and 4 are not unlike those which could be produced by weak seminal acid phosphatase activity. Therefore, fecal phosphatase in semen-free anal swabs analyzed by the method of Emes and Wraxall (4) could, in certain cases, be mistaken for seminal acid phosphatase. Conversely, forensic scientists might also feel justifiably skeptical about confirming the presence of seminal acid phosphatase on anal swabs which are, in fact, stained with aspermatic semen.

Unfortunately, the attempt to eliminate the fecal phosphatase bands by substituting α-naphthyl acid phosphate for MUP in the reaction mixture proved unsuccessful in these experiments. The intensity of the fecal phosphatase band developed with α-naphthyl acid phosphate is visually comparable to the intensity of the fecal phosphatase band developed with MUP.

Based on Sensabaugh's observation (5) that low molecular weight acid phosphatases are unreactive with α-naphthyl acid phosphate, it would appear that the fecal phosphatase detected in this experiment does not fall within the low molecular weight classes of acid phosphatases described by him.

Conclusions

It has been shown that in some instances fecal phosphatase can exhibit the same mobility as seminal acid phosphatase in acrylamide gel electrophoresis. This phenomenon can impede the identification of seminal acid phosphatase on aspermatic anal swabs.

Because the acrylamide gel electrophoresis method of Emes and Wraxall has proved in our experience to be a simple and valuable analytical technique for distinguishing seminal acid phosphatase from vaginal acid phosphatase, further investigation into the problem associated with fecal phosphatase is warranted.

Acknowledgment

The authors are grateful to Daniel Garner for presenting this paper at the 89th Annual Meeting of the AOAC.

References

(1) Davies, A. M., & Wilson, E. M. (1974) *Forensic Sci.* 3, 45–55
(2) Baxter, S. J. (1973) *Med. Sci. Law* 13, 155–165
(3) Lam, K. W., Li, O., Li, C. Y., & Yam, L. T. (1973) *Clin. Chem.* 19, 483–487
(4) Adams, E. G., & Wraxall, B. G. D. (1974) *Forensic Sci.* 3, 57–62
(5) Sensabaugh, G. F. (1975) in *Isozymes*, Vol. 1, C. L. Markert (Ed.), Academic Press, New York, NY, pp. 367–380

Received August 28, 1975. Resubmitted July 13, 1976.

Systematic Approach to the Detection of Explosive Residues. IV. Military Explosives

CHARLES R. MIDKIFF, JR. and WILLARD D. WASHINGTON
Bureau of Alcohol, Tobacco and Firearms, Department of the Treasury, Washington, DC 20226

Part IV of the series encompasses tests for the detection and identification of military explosives collected at the scene of a criminal bombing. Major categories and typical formulations of some common military explosives are described. Tests are described and evaluated for the identification of 2,4,6-trinitrotoluene, cyclotrimethylenetrinitramine, and pentaerythritol tetranitrate. Thin layer chromatography and infrared spectroscopy are used for the identification of major explosive components. Tests for minor components to enable characterization and to distinguish similar compositions are included.

This paper is the fourth in a series describing techniques applicable to the laboratory examination of small amounts of explosives. It focuses primarily on the techniques used in the headquarters laboratory of the Bureau of Alcohol, Tobacco and Firearms for the examination of physical evidence submitted in connection with the investigation of bombing incidents. Methods for the detection and identification of the more common military explosives will be described.

Military explosives are used in only a few per cent of the criminal bombings examined in our laboratory. This is probably attributable to the lesser availability of military explosives as compared with commercial or improvised types.

In general, military explosives are based on the high explosives 2,4,6-trinitrotoluene (TNT) and cyclotrimethylenetrinitramine (RDX) and may be single component or composites (Table 1). Pentaerythritol tetranitrate (PETN) is also included in this paper since it is the principal explosive in both military and commercial detonating cord and sheet explosives.

Military dynamite (Table 1), which differs from commercial dynamite by containing no nitroglycerin component or any inorganic nitrate salts, is a medium velocity (20,000 fps) plastic explosive which is packaged in standard dynamite cartridge waxed-paper wrappers. Three sizes are available: M1—1¼ × 8″, M2—1½ × 8″, and M3—1½ × 12″. Military dynamite is equivalent in strength to 60% straight commercial dynamite but it is safer to handle and store. The other composite explosives listed are used in sizes and configurations dictated by their application, ranging from hand grenade fillers to cratering charges.

Experimental

Apparatus

See description of apparatus in refs. 1–3 plus the following:

(a) *X-Ray diffractometer.*—Diano power source, Rigaku wide angle goniometer, and copper diffraction tube.

(b) *Diamond cell sample solder.*—For infrared samples (manufactured by High Pressure Diamond Optics Inc., 929 Mackall Ave, McLean, VA 22101).

(c) *Beam condenser.* — 4× for Perkin-Elmer Model 621 infrared spectrometer.

(d) *Thin layer plates.*—Silica gel G, 0.25 mm on glass (Analtech).

Table 1. Types of military explosives[a]

Designation	High explosive, %	Binder/additives, %	Color
Amatol (various formulations)	TNT–20,40,50,55,60	ammonium nitrate–80,60,50,45,40	yellow-brown to light yellow
Composition A	RDX, 91	beeswax, 9	white to buff
Composition B	RDX-TNT, 60+40	none, 3% wax	light yellow to brown
Composition C 1, 2, and 3	RDX-Tetryl-TNT, 71+3+4	plasticizing oil-DNT-MNT, 6+10+5	yellow to brown
Composition C-4	RDX, 91	oil-DOS-PIB, 1.6+5.3+2.1	white
Military dynamite	RDX-TNT, 75+15	oil and PIB-cornstarch, 5+5	white

[a] Notations: DNT = dinitrotoluene, DOS = di-(2-ethylhexyl) sebacate, MNT = mononitrotoluene, PIB = polyisobutylene, RDX = cyclotrimethylenetrinitramine, TNT = 2,4,6-trinitrotoluene, Tetryl = methylpicrylnitramine.

Table 2. Reported TLC systems for TNT

Plate[a]	Eluant	Overspray	R_f TNT	R_f DNT	Ref.
A	benzene-cyclohexane-ethyl acetate (50+45+5)	EDA/UV light	—	—	4
A	benzene-ethyl ether-ethanol (50+30+20)	EDA/UV light	—	—	4
B	ethyl acetate-petroleum ether (15+85)	*p*-DEAB[b]	—	—	5
B	1,2-dichloroethane-petroleum ether (25+75)	*p*-DEAB[b]	—	—	5
C	trichloroethylene-acetone (80+20)	EDA/DMSO, *o*-tolidine[c]	0.59	0.58	Zitrin
D		KOH (ethanol), Griess	0.60	—	6
E	benzene-nitromethane (3+1)	none/254 nm UV	0.88	—	7
E	Toluene-benzene-*n*-hexane-*n*-pentane-acetone (5+5+4+1+1)	none/254 nm UV	0.70	—	7
F	benzene-hexane-pentane (50+40+10)	none/254 nm UV	0.28	—	8
F	benzene-hexane-pentane-acetone (50+40+10+10)	none/254 nm UV	0.83	—	8
G	benzene	$TiCl_3$/DMAB[d]	0.44	0.40	9
E		UV light	0.68	N/A	7
H		DPA (ethanol)/UV/H_2SO_4	0.75	0.70	10
I		$TiCl_3$/DMAB[d]	0.40	0.37	11
H	benzene-hexane (1+1)	DPA/UV/H_2SO_4	0.45	0.50	10
I		$TiCl_3$/DMAB[d]	0.20	0.17	11
H	xylene-hexane (3+2)	DPA/UV/H_2SO_4	0.40	0.40	10
I		$TiCl_3$/DMAB[d]	0.30	0.22	11
H	carbon tetrachloride-1,2-dichloroethane (4+1)	DPA/UV/H_2SO_4	0.45	0.50	10
I		$TiCl_3$/DMAB[d]	0.15	0.20	11
H	acetone-chloroform (1+1)	DPA/UV/H_2SO_4	0.90	0.95	10
I		$TiCl_3$/DMAB[d]	0.67	0.63	11
H	hexane-acetone (4+1)	DPA/UV/H_2SO_4	0.40	0.40	10
H	chloroform	DPA/UV/H_2SO_4	0.75	0.80	10
I	ethanol	$TiCl_3$/DMAB[d]	0.61	0.65	11

[a] A = silica gel/starch on glass plates; B = silica gel/zinc on glass plates; C = silica gel, 0.2 mm on aluminum sheet; D = silica gel (E. Merck); E = silica gel on polyethylene terephthalate (Brinkmann); F = silica gel; G = silica gel, 0.25 mm on glass plates; H = silica gel, 0.25 mm on glass plates (Analtech); I = silica gel, 0.25 mm on glass plates (Mackery-Nagel).

[b] *p*-Diethylaminobenzaldehyde (DEAB), 0.25% in absolute ethanol made 0.25*N* HCl with HCl.

[c] Ethylene diamine (EDA) and dimethylsulfoxide (equal volumes) or *o*-tolidine (5% in 95% ethanol).

[d] $TiCl_3$ (15% in HCl) spray, air dry, spray with 1 g *p*-dimethylaminobenzaldehyde (DMAB) in 30 ml ethanol, 3 ml HCl (1+19), and 180 ml *n*-butyl alcohol.

Procedure

Following examination by the vapor trace analyzer (1) for presence of commercial dynamite, debris from suspected bombing is examined microscopically for particles of unburned explosive. Any particles observed are manually removed for further testing (2). Composite military explosives may be recognizable as white-to-brown putty-like substances. If no explosive particles are recognized, bulk sample is extracted with $CHCl_3$ and then with dry acetone, and extracts are concentrated as previously described (3) before further testing. Separate, concentrated extracts are examined by thin layer chromatography (TLC) and/or infrared (IR) spectroscopy.

Results and Discussion

Identification of 2,4,6-Trinitrotoluene

TNT is a colorless solid which melts at 81°C when pure. The normal military product ranges from light yellow to brown. It is encountered as flakes, prills, crystals, or cast solid. In our laboratory, TNT is identified by spot tests, TLC, IR, and x-ray diffraction (XRD). When particles of suspected TNT are noted, a sample is placed in a spot plate and several drops of a saturated solution of potassium hydroxide in ethanol are added. TNT gives an immediate deep red color which persists, whereas 2,4-dinitrotoluene (2,4-DNT) gives an immediate blue color which slowly changes to a dark brown. No color reaction is obtained with tetracene, PETN, RDX, or cyclotetramethylenetetranitramine (HMX).

Thin layer chromatography is applicable to both identification and comparison of known and questioned TNT samples. The questioned

Received April 26, 1976.

Table 3. Evaluation of TLC systems for TNT/DNT[a]

Eluant	R_f TNT	R_f DNT
Ethanol	0.90	0.87
Benzene	0.85	0.80
Benzene-hexane (1+1)	0.45	0.45
Xylene-hexane (3+2)	0.60	0.50
Carbon tetrachloride-dichloroethane (4+1)	0.40	0.50
Acetone-chloroform (1+1)	0.95	0.95
Hexane-acetone (4+1)	0.50	0.55
Chloroform	0.85	0.90
Benzene-hexane-pentane (5+4+1)	0.45	0.45
Dichloroethane-petroleum ether (1+3)	0.25	0.45
Benzene-hexane-pentane-acetone (5+4+1+1)	0.95	0.90
Ethyl acetate-petroleum ether (15+85)	0.75	0.65
Toluene-benzene-hexane-pentane—acetone (5+5+4+1+1)	0.85	0.75

[a] Oversprayed with DPA in ethanol, dried, sprayed with KOH in ethanol: DNT is yellow, TNT is red.

material is dissolved in chloroform and spotted on a TLC plate along with a solution containing known TNT. A number of solvent systems have been reported for the TLC examination of TNT (4–11) (Table 2). R_f values for 2,4-DNT are presented for comparison because it is among the common minor components of military TNT. used. The identification of these additional compounds is of value when 2 or more samples are being compared.

From the data in Table 2 it can be seen that wide variations exist in the reported R_f values. The work of Chandler *et al.* (4), Yasuda (5), and Bilson (12) describes the identification of several additional compounds present in military TNT and their colors with the spray reagents even when the same support and solvent system are used. A number of the solvent systems shown were tested to determine their efficiency in resolving TNT and DNT and to establish the expected R_f values with the TLC plates routinely used in our laboratory which are not activated or otherwise pretreated. Results are shown in Table 3. It is apparent that significant effects are exerted on the R_f values obtained as a function of plate type, support thickness, and pretreatment. These variations emphasize the necessity for simultaneous examination of known and questioned explosive samples on the same plate. Of the systems tested, the best resolution was obtained with dichloroethane-petroleum ether (1+3); however, satisfactory resolution was also obtained with carbon tetrachloride-dichloroethane (4+1) and, in view of its value for TLC of commercial dynamites (3), it is the system of choice for screening in our laboratory.

IR spectroscopy is the best method for confirmation when sufficient sample or extract is available. Solid material can be examined as a KBr pellet (preferably ground under hexane) as shown in Fig. 1, or as a solution in chloroform with chloroform as reference, as shown in Fig. 2. Small particles (less than 1 mg) can be examined directly by mounting in a diamond cell sample holder and very sharp IR spectra are obtained (Fig. 3). TNT is also soluble in acetone; however, some difficulties are encountered with this solvent. Moisture absorption by the acetone results in water band interference in the IR spectrum. This problem is avoided by slowly evaporating the acetone solution to dryness and dissolving the residue in chloroform before IR examination. Other problems encountered involve more complex solvent interactions. When samples of TNT from several sources were dissolved in acetone, the solutions became light pink, and the color slowly deepened on standing. While the initial color may be attributed to the presence of by-products in the impure material (4), the intensification of color after solution is complete suggests solvent interactions. With acetone as solvent, several bands in the IR spectrum are suppressed compared with the spectrum in benzene, carbon tetrachloride, or chloroform. This distortion (especially notable at 1300–1400, 900, and 950 cm^{-1}) may make identification difficult particularly when very dilute acetone solutions of the explosive are being examined. This is shown in Fig. 4. Collections of IR spectra for a number of explosives are given by Pristera *et al.* (13) and Chason and Norwitz (14). Newer instruments and other solvents may provide somewhat better resolution than those in ref. 14, but major band assignments for explosive identification remain essentially unchanged. When only small volumes of test solution are available, the IR spectrum can be obtained and the solution from the IR cell can be used for TLC. Alternatively, the test solution can be evaporated onto a KBr disk and covered with another disk for IR examination. The solid material is washed off the KBr disk for TLC (11).

Some preliminary attempts have been made

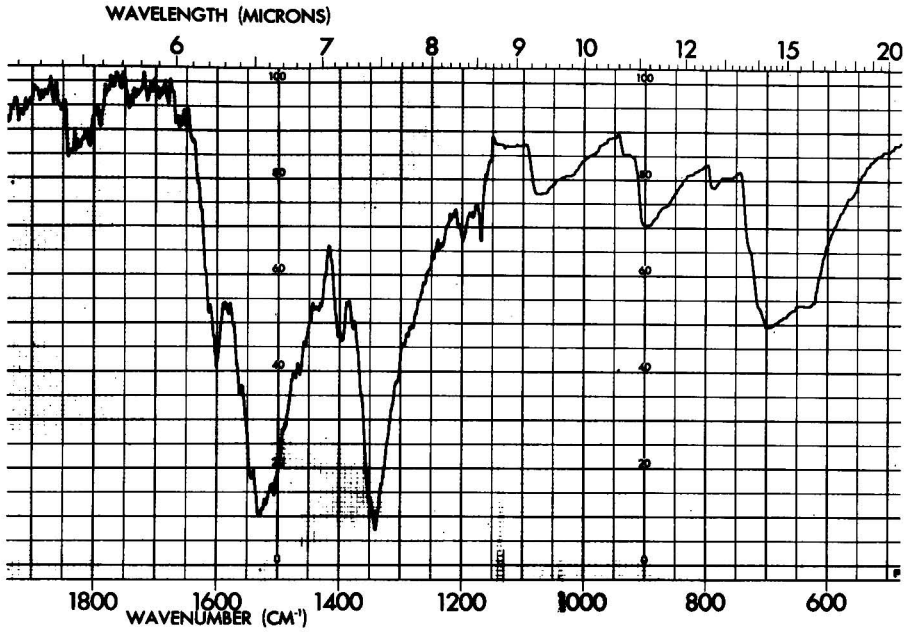


FIG. 1—IR spectrum of 5 mg TNT/50 mg KBr (ground under hexane).

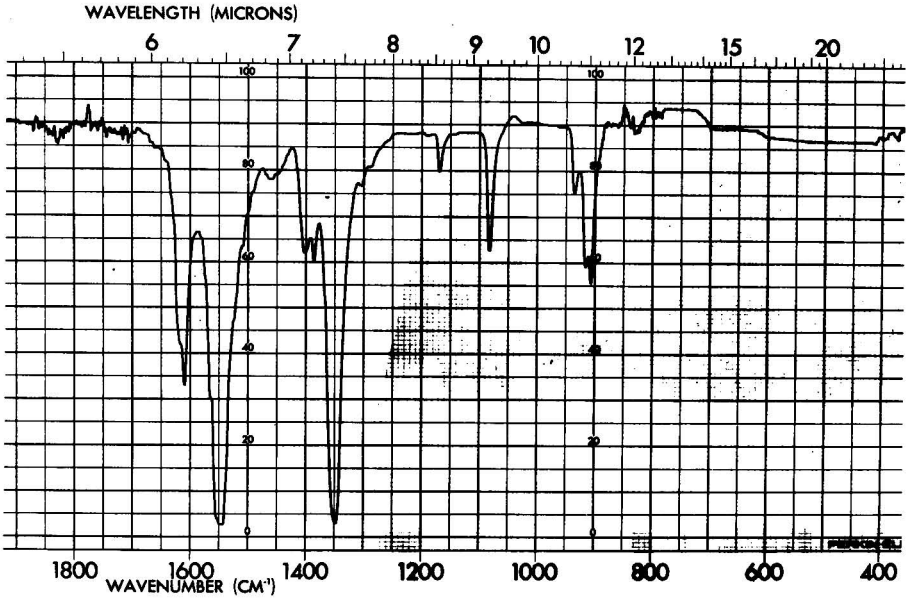


FIG. 2—IR spectrum of 30 mg TNT/2 ml $CHCl_3$.

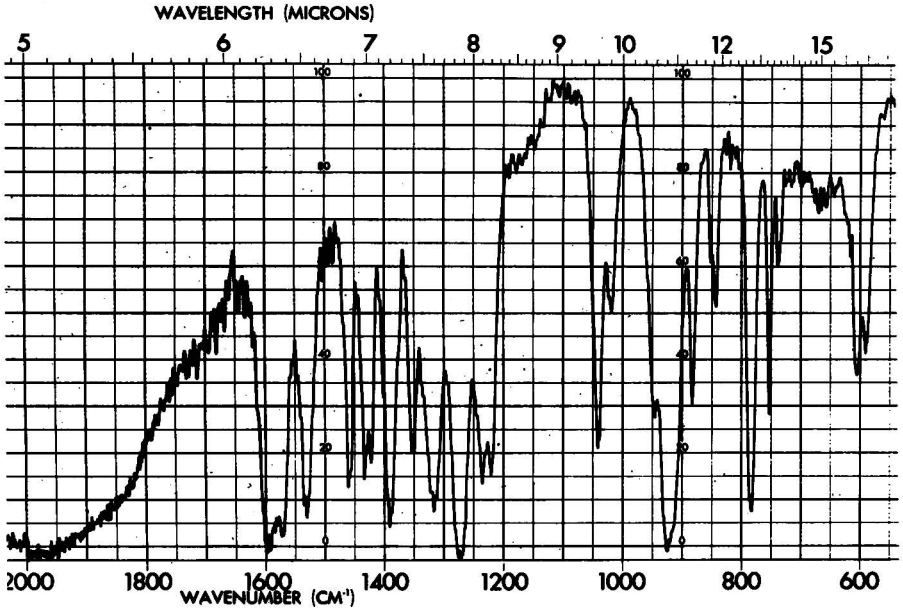


FIG. 3—IR spectrum of TNT in diamond cell.

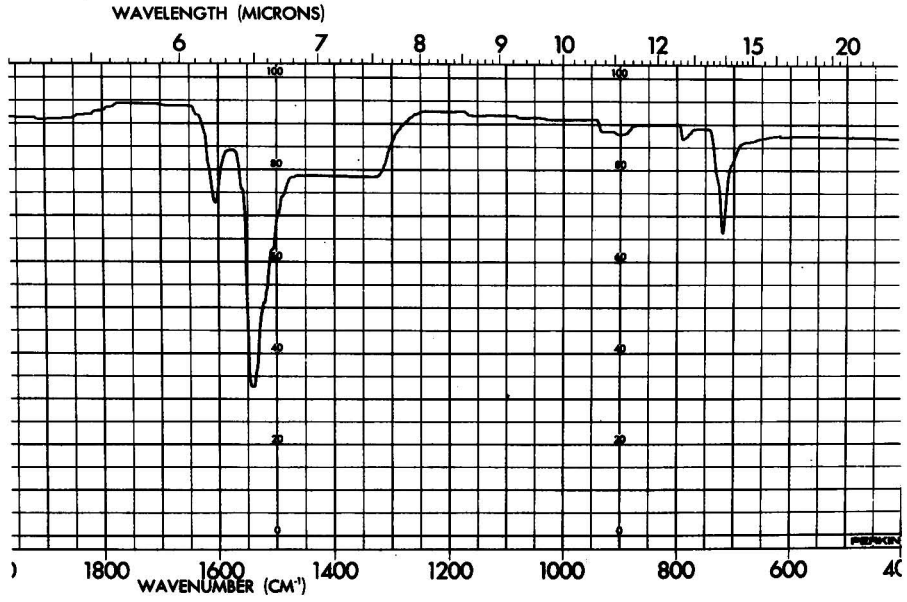


FIG. 4—IR spectrum of TNT in acetone.

in our laboratory to determine TNT in solutions by gas-liquid chromatography (GLC); however, no detailed studies have been made. The use of GLC on glass columns has been reported for the examination of traces of a number of explosives in extracts of bombing debris, using both flame ionization and electron capture detection (S. Zitrin, Weizman Institute for Science, Rehovot, Israel, 1974). The latter system has also been applied to the examination of explosive compounds separated as spots on a thin layer chromatogram (8). High-pressure liquid chromatography (HPLC) provides an additional potential technique for the detection of TNT in solvent wash solutions since it avoids possible thermal decomposition of explosive compounds. The separation of DNT and TNT from each other and from other explosives present in composites has been reported (15). HPLC will be evaluated for examination of extracts during the continuation of this work. Preliminary work by Saferstein *et al.* (16) indicated that chemical ionization mass spectrometry (CI-MS) may be suitable for the detection of explosive residues in extracts of debris material. Mass spectral data were obtained for several explosives in addition to TNT and the examination of debris extract samples is planned. Zitrin and Yinon (17) have recently reported the detection of TNT by CI-MS in an acetone extract of residues from an explosion site.

A capability for detecting trace concentrations of TNT vapor in air would allow the rapid laboratory screening of debris for military explosives in the same manner as presently used for commercial dynamite. While it has been contended that several commercial instruments have this capability, field tests by our laboratory using these instruments have generally been unsatisfactory. Among the techniques proposed by instrument manufacturers for TNT vapor detection are gas chromatography by vapor trace analyzer (1) and by mass spectrometry (18). A newer approach is plasma chromatography (19, 20) which appears to offer sensitivity in the nanogram range. Several versions of the gas chromatographic vapor detectors have been evaluated in our laboratory and lacked sufficient sensitivity to reliably detect the amounts of TNT expected to be present in post-blast evidence. Similar results have been obtained with material collected following test detonations involving military explosives. Neither the mass spectrometer nor plasma chromatograph type detectors have been evaluated by us.

Identification of Cyclotrimethylenetrinitramine

RDX is most often encountered as a component of military dynamite or one of the other composite explosives, e.g., C-4. Two spot tests are useful for preliminary identification of RDX alone or admixed. In the thymol test, a few milligrams of the sample is placed in a test tube with 200 mg thymol and 6 drops of concentrated sulfuric acid. The tube is warmed 5 min at 100°C, and 5–10 ml ethanol is added. A blue color is produced by RDX (21). This procedure is a modification of an earlier version (22) which was designed to eliminate interference from sugars, aldehydes, and most other nitramines. By repeating the test at 150°C, RDX can be distinguished from HMX which gives an olive color (21). Other versions of the test do not specify heating the sample before adding alcohol (23). A second spot test uses a 0.4% solution of 6-amino-1-naphthol sulfonic acid (J-acid) in concentrated sulfuric acid. A few drops of the J-acid reagent are added to the questioned sample in a spot plate. The acid will turn yellow-red if RDX is present. After a few seconds, 95% ethanol is added dropwise. An immediate blue color is produced which proceeds to a blue-green and finally fades to gray. A similar test has been reported with pharmaceutical nitroglycerin tablets and J-acid (24). For confirmation purposes both tests should be performed and the identification should be confirmed by TLC and/or IR spectroscopy.

As manufactured, RDX often contains small amounts of HMX. The quantitative determination of the HMX present can provide one basis for the comparison of 2 samples suspected of having a common origin. RDX and HMX may be detected by spot tests (25, 26) or by their brown-ring reaction with ferrous sulfate in sulfuric acid (27); however, a number of other nitro compounds interfere. This reaction has been reported for the quantitative spectrophotometric determination of small amounts of HMX in RDX (28) and, when sufficient sample is available, could be of value in the comparison of known and questioned samples to determine possible common origin. It has not been used

Table 4. TLC systems for RDX and HMX

Plate[a]	Eluant	Overspray	R_f RDX	R_f HMX	Ref.
A	chloroform-acetone (1+1)	thymol[b]	0.47	0.40	9
B		DPA/UV/H_2SO_4	0.7	—	10
C		NaOH/Griess[c]	0.46	—	11
B		DPA(ethanol)/UV/H_2SO_4	0.95	0.85	[d]
B		thymol/H_2SO_4[e]	0.73	0.64	[f]
B		CTA/H_2SO_4[g]	0.73	0.64	[h]
D	1,2-dichloroethane-acetonitrile (90+10)	Griess	0.58	0.25	Zitrin
E	benzene-nitromethane (1+1)	none/254 nm UV	0.80	0.70	7
E	benzene-nitromethane (3+1)	none/254 nm UV	0.51	0.24	7
B	chloroform-acetone (2+1)	DPA(ethanol)/UV	0.80	0.55	[d]
B	chloroform-acetone-ethanol (1+1+1)	DPA(ethanol)/UV	1.0	1.0	[d]
B	ethyl acetate-petroleum ether (15+85)	DPA(ethanol)/UV	0.05	0.00	[d]
B	hexane-acetone (4+1)	DPA/UV/H_2SO_4	0.2	—	10
B		DPA(ethanol)/UV	0.05	0.00	[d]
C	ethanol	NaOH/Griess	0.49	—	11
B		DPA(ethanol)/UV	0.70	0.0	[d]
B	chloroform	DPA/UV/H_2SO_4	0.15	—	10
B		DPA(ethanol)/UV	0.20	0.03	[d]
B	carbon tetrachloride-1,2-dichloroethane (4+1)	DPA/UV/H_2SO_4	0.02	—	10
C		NaOH/Griess	0.0	—	11
B	benzene-hexane (1+1)	DPA/UV/H_2SO_4	0.02	—	10
C		NaOH/Griess	0.0	—	11
B	xylene-hexane (3+2)	DPA/UV/H_2SO_4	0.03	—	10
C		NaOH/Griess	0.0	—	11
B	benzene	DPA/UV/H_2SO_4	0.10	—	10
C		NaOH/Griess	0.0	—	11
F	trichloroethylene-acetone (4+1)	DPA(ethanol)/UV	0.16	—	6
D		KOH/Griess	0.15	0.05	Zitrin

[a] A = silica gel, 0.25 mm on glass plates; B = silica gel, 0.25 mm on glass plates (Analtech); C = silica gel, 0.25 mm on glass plates (Mackery-Nagel); D = silica gel, 0.2 mm on aluminum sheet; E = silica gel on polyethylene terephthalate (Brinkmann); F = silica gel (E. Merck).

[b] 1.5 g thymol in 10 ml H_2SO_4 spray, dry 10 min at 100°C.

[c] Equal volumes of 1 and 2 mixed just before use: 1 = 0.5 g sulfanilic acid in 50 ml acetic acid and 100 ml water; 2 = 0.1 g 2-naphthylamine in 120 ml hot water, cool, and add 30 ml acetic acid.

[d] Unpublished work from this laboratory.

[e] 15% thymol in 95% ethanol, overspray with H_2SO_4, dry at 105°C for 5 min.

[f] R. Holmes, Bureau of Alcohol, Tobacco and Firearms, Philadelphia, PA, 1975.

[g] Chromotropic acid (CTA) 2% (aq.) overspray with H_2SO_4, dry at 105°C for 5 min.

[h] J. Chrostowski, Bureau of Alcohol, Tobacco and Firearms, Philadelphia, PA, 1975.

in our laboratory because it requires a larger sample than is generally available. The determination of HMX in composite explosives (29) and of HMX and RDX (30) by gel permeation chromatography has been reported. This method could be applicable to the examination of evidentiary samples of composite explosives such as C-4 when 50–60 mg material is available. Both RDX and HMX (29) and RDX and PETN in composite explosives have been determined by HPLC, using an ultraviolet detector (J. Strimaitis, Waters Associates, Inc., Milford, MA, 1975); however, no work has been done on HPLC of explosives in our laboratory.

TLC provides confirmation of RDX and HMX in explosive formulations. Systems applicable to both RDX and HMX are shown in Table 4. In general, solvent systems used for cyclic nitramines are different from those suitable for nitroaliphatics or aromatics. Beveridge *et al.* (11) and Parker *et al.* (10) tested a number of systems which, while suitable for TNT, gave very poor migration for RDX. They report satisfactory resolution of RDX and TNT with acetone-chloroform (1+1); however, neither group included values for HMX which are important in RDX explosive examinations. Using the same solvent system with our plates, TNT, RDX, and HMX were poorly resolved. Ethanol or chloroform alone were not satisfactory TLC solvents for RDX and HMX because they gave no appreciable migration of HMX from the origin. The best solvent found for RDX and HMX was chloroform-acetone

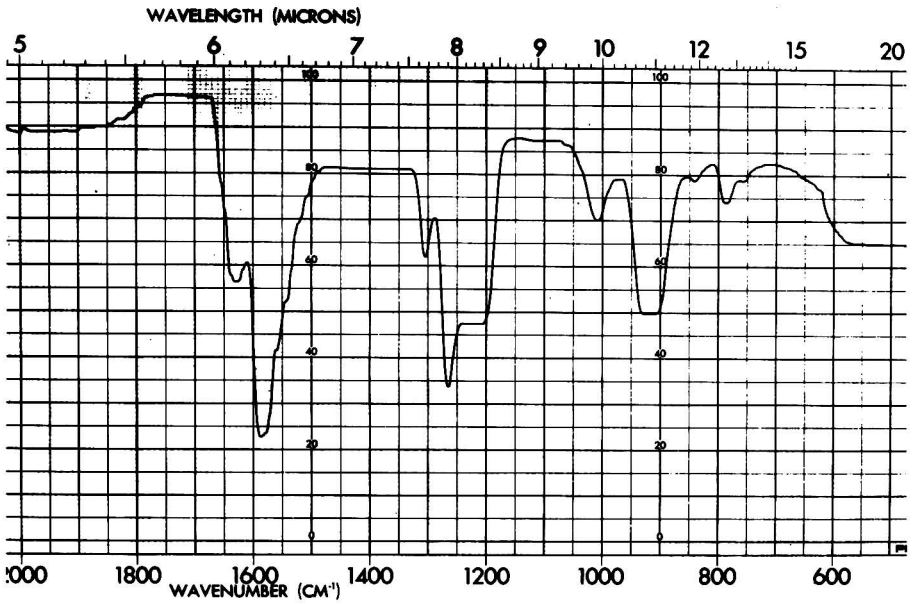


FIG. 5—IR spectrum of 15 mg RDX/ml acetone.

(2+1) which allows HMX present in RDX and vice versa to be easily seen upon visualization. With this system as well as several of the others tested, HMX tends to form diffuse spots or to streak. We plan to do further development work on suitable TLC systems for military explosives.

In contrast with TNT, HMX and RDX are readily identified in dry acetone solution by IR spectroscopy. These compounds are soluble in acetone and allow the use of this solvent without the band suppression found for TNT. If sufficient explosive is available, a KBr pellet may be prepared for IR examination or the diamond cell may be used directly. IR spectra of RDX and HMX are shown in Figs. 5–10.

X-ray powder diffraction provides a convenient method for the identification of suspected RDX or HMX. RDX is included in the 1971 ASTM powder diffraction file (File No. 5-576) as is TNT (File No. 13-779). HMX is not listed in the 1971 file but is among a number of patterns of explosives by Alston *et al.* (31).

Identification of Pentaerythritol Tetranitrate

PETN is the main explosive in detonating cord used to initiate high explosives and is also occasionally used in M118 Flex-X type explosives. While it has been reported that PETN may be detected from residues of detonating cord (11), our experience, both with tests and actual case samples, is that it is unlikely to be found in significant quantities in bomb debris. It is more often necessary to confirm the identity of this explosive in a portion of undetonated cord found at a bombing scene or obtained from a suspect. In our laboratory IR, TLC, and XRD are suitable for this purpose. IR spectra of PETN as a KBr pellet, with the diamond cell, and in acetone solution, are shown in Figs. 11–13; TLC systems reported for PETN are shown in Table 5.

It had been observed in our laboratory that with the TLC systems in routine use, the separation of PETN and nitroglycerin (NG) is not effective. This is also evident in Table 5 where R_f values reported for both PETN and NG are shown; differences, in most instances, are quite small. Operationally, little difficulty is encountered because U.S. commercial dynamite commonly contains both ethylene glycol dinitrate (EGDN) and NG and both are observed on the TLC plate. The reported TLC systems were evaluated and other potential systems were studied to identify a satisfactory system for the

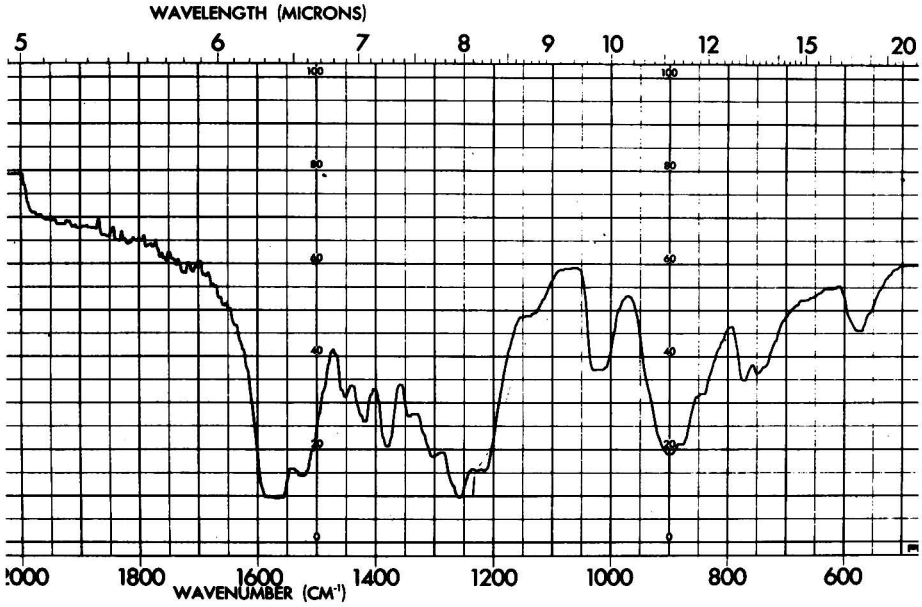


FIG. 6—IR spectrum of 1 mg RDX/50 mg KBr.

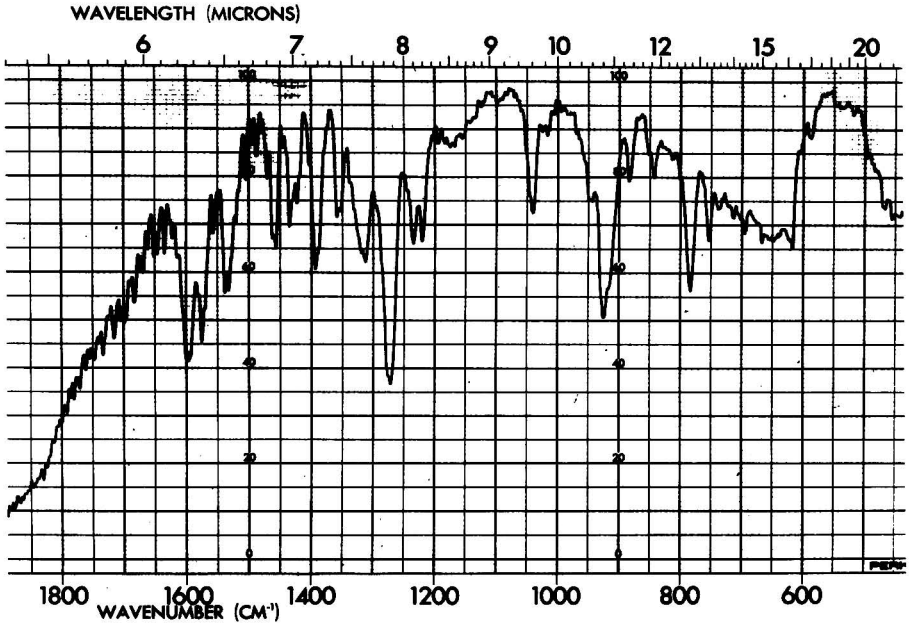


FIG. 7—IR spectrum of RDX in diamond cell.

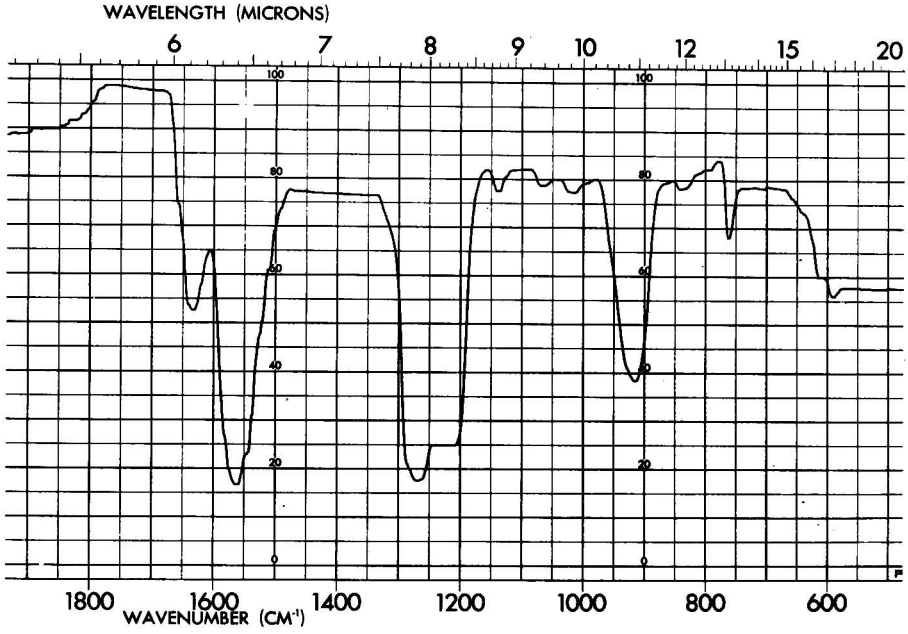


FIG. 8—IR spectrum of 15 mg HMX/ml acetone.

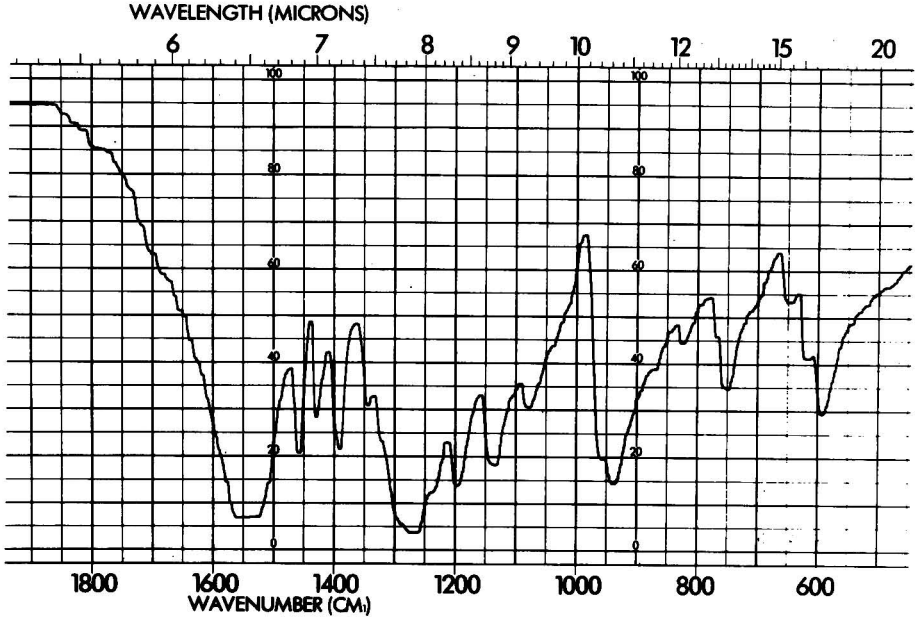


FIG. 9—IR spectrum of HMX (ground under hexane).

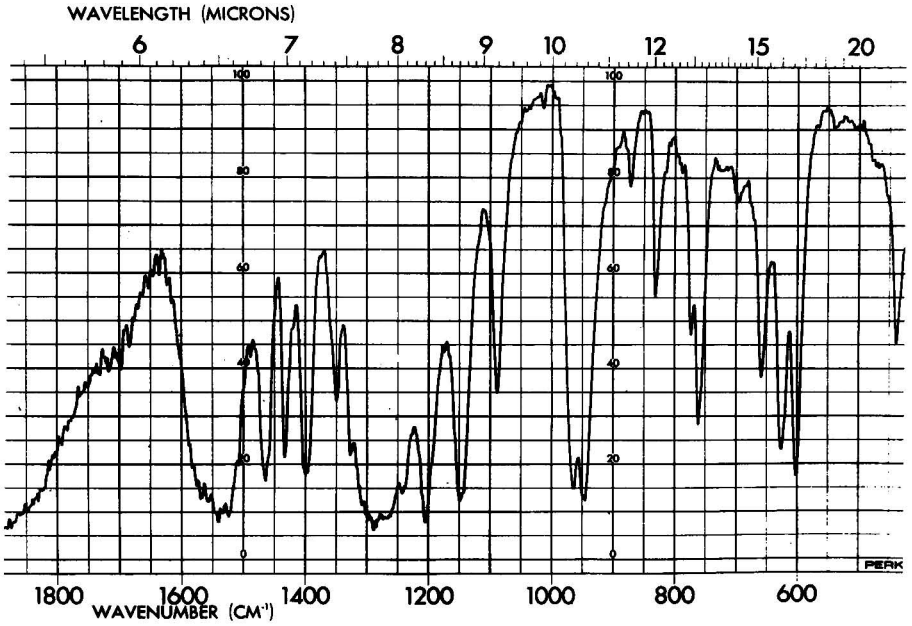


FIG. 10—IR spectrum of HMX in diamond cell.

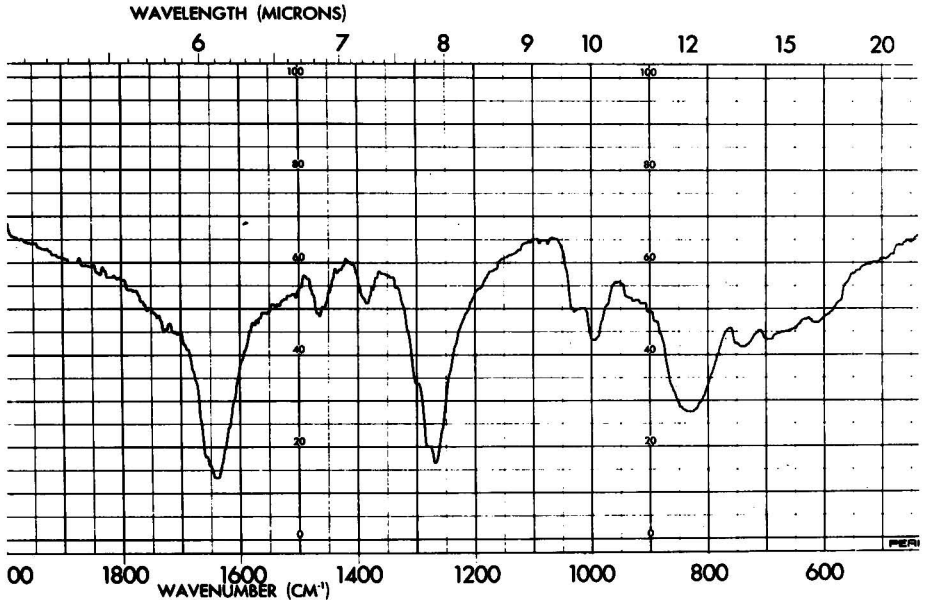


FIG. 11—IR spectrum of 1 mg PETN/50 mg KBr (ground under hexane).

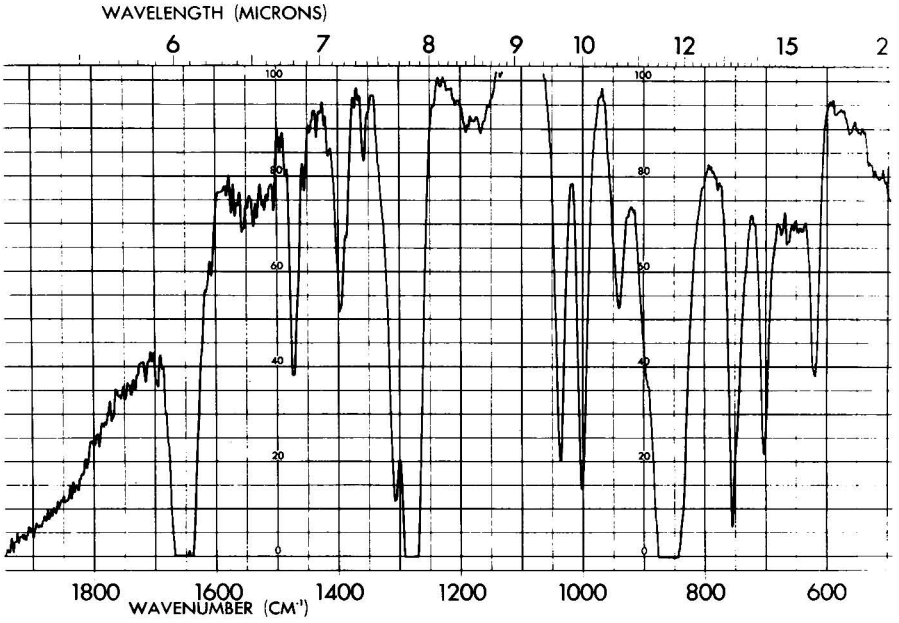


FIG. 12—IR spectrum of PETN in diamond cell.

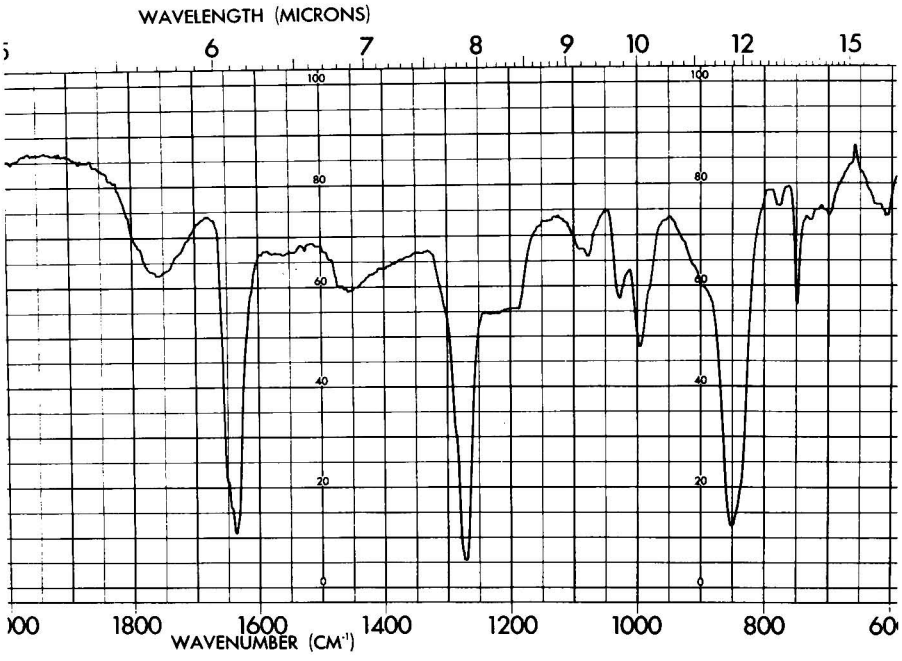


FIG. 13—IR spectrum of 15 mg PETN/ml acetone.

Table 5. Reported TLC systems for PETN and NG

Plate[a]	Eluant	Overspray	R_f PETN	R_f NG	Ref.
B	chloroform-acetone (1+1)	DPA/UV/H_2SO_4	0.95	0.90	10
C		NaOH/Griess	0.71	0.65	11
A		NaOH/Griess	0.69	0.38	9
B	chloroform	DPA/UV/H_2SO_4	0.7	0.7	10
B	benzene	DPA/UV/H_2SO_4	0.65	0.65	10
C		NaOH/Griess	0.38	0.37	11
B	benzene-hexane (1+1)	DPA/UV/H_2SO_4	0.30	0.35	10
C		NaOH/Griess	0.15	0.17	11
B	xylene-hexane (3+2)	DPA/UV/H_2SO_4	0.30	0.35	10
C		NaOH/Griess	0.21	0.21	11
D		DPA(ethanol)/UV	0.37	0.46	32
B	carbon tetrachloride-1,2-dichloroethane (4+1)	DPA/UV/H_2SO_4	0.30	0.35	10
C		NaOH/Griess	0.16	0.12	11
C	ethanol	NaOH/Griess	0.64	0.60	11

[a] Plates A, B, and C, same as in Table 4; Plate D = alumina (Eastman 6063).

examination of samples which could contain both NG and PETN. Results are shown in Table 6. No completely satisfactory single system was found but in most of the systems PETN tended to streak rather than to give a well defined spot. When streaking is observed in a screening solvent system, e.g., carbon tetrachloride-dichloroethane (4+1), the questioned sample should be re-examined by using either a different plate or solvent system. Mixtures such as carbon tetrachloride-chloroform (1+1) or dichloroethane-petroleum ether (1+2) give typical R_f values for PETN and NG quite different from those in the screening system. PETN gives the typical spot test reactions for nitrates, i.e., deep blue with diphenylamine in sulfuric acid and red with the modified Griess reagent. The use of HPLC (Strimaitis) and GLC and polarographic techniques (33) for the detection and identification of PETN has been reported but has not been studied in our laboratory. A useful discussion of the properties and manufacture of PETN is found in Urbanski (34).

Characterization of Composites

Composite explosives can be identified based on solubility and chemical tests. A sample of a suspected composite is extracted several times with chloroform or carbon tetrachloride to remove TNT. A portion of the extract is tested by adding 3–4 drops of alcoholic potassium hydroxide. A red color indicates TNT. A second portion of the extract is then examined by TLC or IR spectroscopy to confirm the presence of TNT. The solid residue from the extraction is treated with acetone and Composition B, RDX and PETN, will dissolve completely. RDX and PETN can be identified by TLC of a portion of the acetone extract. IR examination is performed on the remaining acetone solution. Both tests should be performed since PETN alone and mixtures of PETN and RDX have been used in sheet form such as explosive charges in letter bombs and military Flex-X. Some residues will

Table 6. Evaluation of TLC systems for PETN and NG (silica gel plates), DPA (ethanol)/UV

Eluant	R_f PETN	R_f NG
Chloroform	0.70	0.75
Carbon tetrachloride	0.05	0.10
Petroleum ether	0.02	0.04
Ethanol	0.95	0.95
1, 2-Dichloroethane	0.95	0.95
Ethanol (75%)	1.0	1.0
Xylene-hexane (3+2)	0.45	0.45
Carbon tetrachloride-1,2-dichloroethane (4+1)	0.30	0.35
Benzene-hexane (1+1)	0.40	0.45
Chloroform-acetone (1+1)	1.0	1.0
(2+1)	1.0	1.0
Chloroform-acetone-ethanol (1+1+1)	1.0	1.0
Chloroform-carbon tetrachloride (1+1)	0.43	0.52
Petroleum ether-ethanol (4+1)	0.95	0.95
(9+1)	0.70	0.70
1,2-Dichloroethane-petroleum ether (1+1)	0.80	0.85
(1+2)	0.43	0.53
(1+3)	0.25	0.37
Xylene-hexane-chloroform (3+2+1)	0.45	0.45
Xylene-hexane (3+2)	0.45	0.45

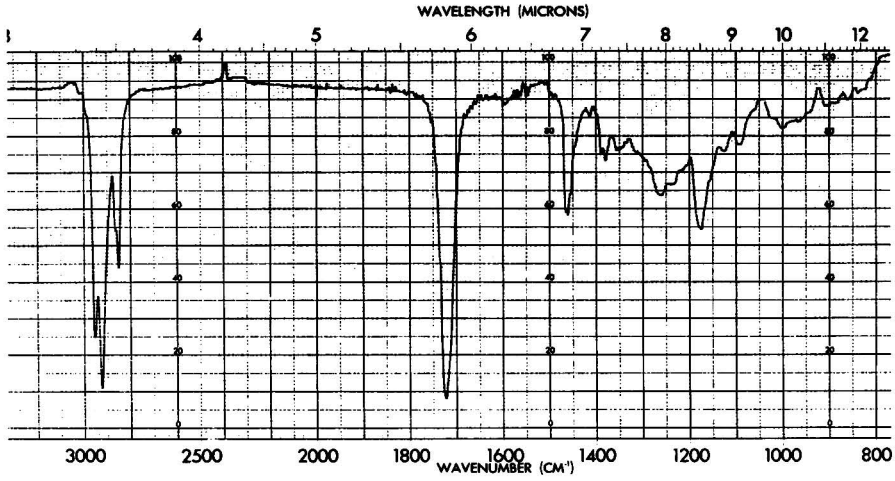


FIG. 14—IR spectrum of DOS in chloroform.

remain following the acetone extraction of Composition A, Composition C, or military dynamite. Composition A can be distinguished from Composition C as the former is dry and crumbles to a powder, whereas Composition C is soft and plastic. A discussion of composite explosive types A–D including variations within a single type is found in Federoff and Sheffield (35), Newhouser (36), and in ref. 37. Appreciable residue of ammonium nitrate will remain following chloroform extraction of Amatols. This can be identified by IR spectroscopy (13), XRD (31), spot tests with Nessler, nitron, or Griess reagents (3), or by TLC in ethanol (11). Composition and properties of various Amatols are considered in Federoff and Sheffield, Vol. 1 (38).

The plastic explosive C-4 is supplied to the U.S. military in only a single grade; its formulation is generally that given in Table 1. For reasons of availability, the plasticizer normally used, di-(2-ethylhexyl) sebacate (DOS), may be replaced with di-(2-ethylhexyl) adipate (DOA). The RDX used may contain up to 10% HMX; British and Canadian plastic explosives use higher purity RDX (ca 99%) and different plasticizers. These variations in formulation can provide a basis for sample comparisons when sufficient material is available. The commonly used plasticizers are soluble in chloroform or carbon tetrachloride and may be extracted for IR examination. The small amounts (ca 4%) of oil and binder present do not interfere. Figures 14–18 show chloroform spectra of solutions of plasticizers and binders and chloroform extracts from several plastic explosives obtained in our laboratory. It should be noted that the explosive used in M118 (Flex-X) detonating sheet varies with manufacturer. Some use PETN and others use RDX. Future manufacture may use other formulations.

X-ray diffraction has been previously mentioned for the identification of base explosives but is also applicable to composites. Alston *et al.* (31) have compiled diffraction patterns of selected explosives and associated ingredients, including 5 which are not included in the 1971 x-ray diffraction file: dinitroxydiethylnitramine (DINA), methyl picrate, ethyl picrate, nitrated ethylnitro oxamide (NENO), and HMX.

Several other works contain background information on composites and describe techniques for their examination and identification. The use of nuclear magnetic resonance has been discussed (39) but has not been evaluated by our laboratory. A work useful to the laboratory analyst by Coates *et al.* (40) contains patterns from infrared, mass spectrographic, and thermal analysis of composite explosives. A series of color tests, in addition to those previously described, suitable for examination and comparison of military explosives is available (41).

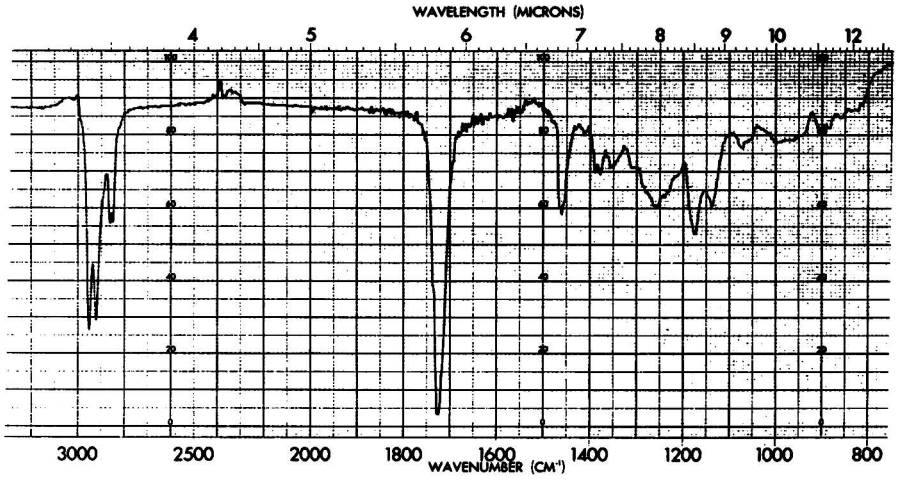


FIG. 15—IR spectrum of DOA in chloroform.

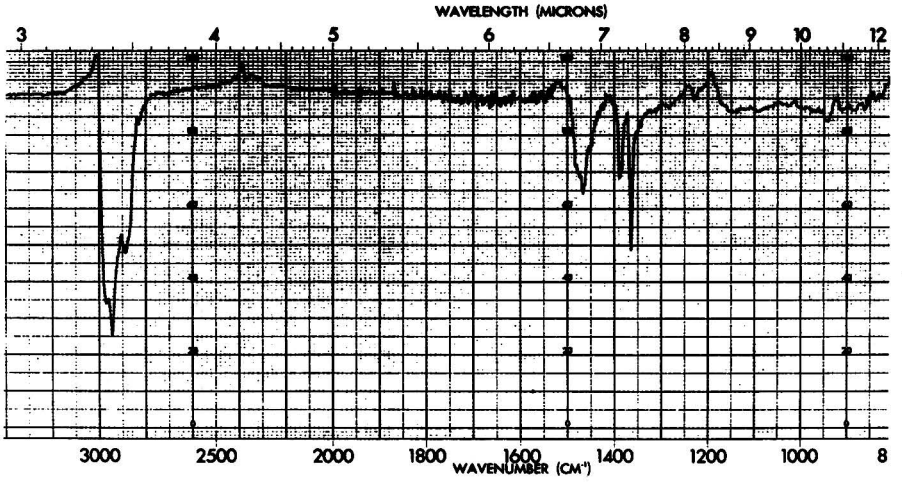


FIG. 16—IR spectrum of PIB in chloroform.

Detection of Military Explosives on Suspects

Since TNT- and RDX-based explosives are of a consistency that makes them easily transferred, they may be detected in a test of the hands of one suspected of having recently handled such materials. A suspect's hands are wrapped with polyester film adhesive tape to collect any adhering particles. The tape is removed and tested by spot tests directly on the tape. Alternatively, cotton swabs moistened with acetone which are used to swab the hands may be re-extracted later with acetone for testing. For tests on the tape, TNT is detected with alcoholic potassium hydroxide, and RDX is detected either by the J-acid method or the thymol test. The thymol reagent is prepared just before use by dissolving 25 mg thymol in 5 ml concentrated sulfuric acid. The reagent is added dropwise to the tape, followed by dropwise addition of ethanol.

Two RDX-based explosives, M118 (Flex-X) and Detasheet, have been reported to give false

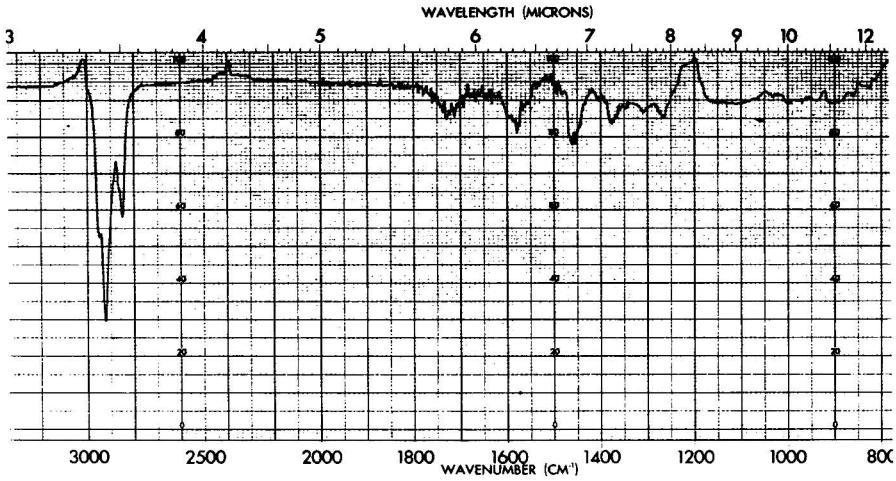


FIG. 17—IR spectrum of PE-4 in chloroform.

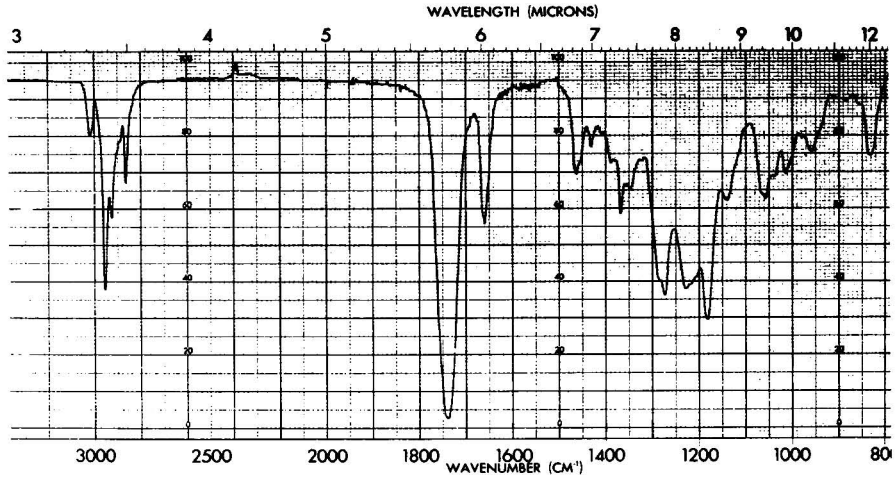


FIG. 18—IR spectrum of Flex-X chloroform extract.

negative tests by the tape lift method (10). When swabs are examined, TLC or IR spectra of acetone extracts provide more convenient and conclusive results than spot tests. Particulate explosives may also be recovered during a visual and microscopic examination of a suspect's clothing (pockets, cuffs, etc.) or vacuumings from a vehicle. These can be examined as previously described. The detection of TNT alone, RDX alone, or both TNT and RDX will provide an indication of the type of military explosive handled by the subject.

Conclusion and Recommendation

From the tests outlined, the analyst should be able to detect and identify most common mili-

This report of the Associate Referee, W. D. Washington, was presented at the 88th Annual Meeting of the AOAC, Oct. 14–17, 1974, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee G and was accepted by the Association. *See* (1975) *JAOAC* **58**, 329.

tary explosives whether present as unexploded particles or in solvent extractions of debris from a bombing scene. From observations of physical and chemical properties and the identification of major explosive components, the particular compositional explosive can be categorized.

Detection of minor components by TLC or IR spectroscopy allows comparison of 2 or more samples suspected of having a common origin. Spot tests or TLC may be used to demonstrate that an individual has recently handled military explosives and to estimate their type. The Associate Referee recommends that study be continued on the topic of Bomb Residues.

Acknowledgments

The authors wish to express their appreciation to staff members for their assistance in this work, in particular to Richard Meyers for TLC, to Karen Walker for typing, and to photographer Don Sumner.

REFERENCES

(1) Washington, W. D., & Midkiff, C. R. (1973) *JAOAC* **56**, 1239–1245
(2) Washington, W. D., & Midkiff, C. R. (1972) *JAOAC* **55**, 811–822
(3) Midkiff, C. R., & Washington, W. D. (1974) *JAOAC* **57**, 1092–1097
(4) Chandler, C. D., Kohlbeck, J. A., & Bolleter, W. T. (1972) *J. Chromatogr.* **64**, 123–128
(5) Yasuda, S. K. (1964) *J. Chromatogr.* **13**, 78–82
(6) Forestier, H., & Helie-Calmet, J. (1974) *Int. Crim. Police Rev.* No. 227, 99–106
(7) Fisco, W. (1975) *J. Forensic Sci. Soc.* **20**, 141–148
(8) Hoffsommer, J. C. (1970) *J. Chromatogr.* **51**, 243–251
(9) Jenkins, R., & Yallop, H. J. (1970) *Explosivstoffe* **6**, 139–141
(10) Parker, R. G., McOwen, J. M., & Cherolis, J. A. (1975) *J. Forensic Sci.* **20**, 254–256
(11) Beveridge, A. D., Payton, S. F., Audette, R. J., Lambertus, A. J., & Shaddick, R. C. (1975) *J. Forensic Sci.* **20**, 431–454
(12) Bilson, K. (1974) "A Qualitative Scheme for the Identification of Some Explosives and Related Compounds by TLC," MQAD Report No. 212, Royal Arsenal, East Woolwich, London SE 18 6TD, United Kingdom
(13) Pristera, F., Halik, M., Castelli, A., & Fredericks, W. (1960) *Anal. Chem.* **32**, 495–508
(14) Chason, D. E., & Norwitz, G. (1972) *Microchem. J.* **17**, 31–60
(15) Doali, J. O., & Juhasz, A. A. (1974) *J. Chromatogr. Sci.* **12**, 51–56
(16) Saferstein, R., Chao, J. M., & Manura, J. J. (1975) *JAOAC* **58**, 734–742
(17) Zitrin, S., & Yinon, J. (1975) "Forensic Identification of Explosives and Drugs by Chemical Ionization Mass Spectrometry," presented at Interpol Meeting, Zurich, Switzerland
(18) Murrmann, R. P., Jenkins, T. F., & Leggett, D. C. (1971) "Composition and Mass Spectra of Impurities in Military Grade TNT Vapor," Special Report 158 AD 725474, USACRRE Laboratory, Hanover, NH
(19) Karasek, F. W., Tatone, O. S., & Kane, D. M. (1973) *Anal. Chem.* **45**, 1210–1214
(20) Karasek, F. W. (1974) *Res. Dev.* **25** (May), 32–36
(21) Amas, S. A. H., & Yallop, H. J. (1969) *Analyst* **94**, 828
(22) Amas, S A. H. & Yallop, H. J. (1966) *J. Forensic Sci. Soc.* **6**, 185
(23) Hoffman, C. M., & Byall, E. B. (1974) *J. Forensic Sci.* **19**, 54–63
(24) Newhouser, C. R. (1972) "Explosives Handling Detection Kit," Technical Bulletin 33–72, National Bomb Data Center, Gaithersburg, MD
(25) Semel, S. (1962) *Chem. Anal.* **51**, 6–7
(26) Feigl, F., & Castrol, D. H. (1962) *Chem. Anal.* **51**, 5–6
(27) Laccetti, M. A., Semel, S., & Roth, M. (1959) *Anal. Chem.* **31**, 1049–1050
(28) Semel, S., Laccetti, M. A., & Roth, M. (1959) *Anal. Chem.* **31**, 1050–1052
(29) Selig, W. (Ed.) (1973) *Some Analytical Methods for Explosives and Explosive Simulants, Part IV,* Publ. UCRL-7873, Lawrence Livermore Laboratory, University of California, Livermore, CA
(30) Freeman, D. H., Angeles, R. M., & Poinescu, I. C. (1976) *J. Chromatogr.* **118**, 157–166
(31) Alston, P. L., Oesterling, D. H., Hartog, L. D., & Shafer, J. E. (1967) *Analysis of Explosives by X-Ray Diffractometry,* Tech. Memo. 247, Naval Ordinance Station, Indian Head, MD
(32) Kempe, C. R., & Tannert, W. K. (1972) *J. Forensic Sci.* **17**, 323–324
(33) Brandone, A., Cerri, T., Marozzi, E., Pariali, A., & Riganti, V. (1975) "Identification of Explosives and Explosive Residues in Investigation of Criminal Assault," presented at Interpol Meeting, Zurich, Switzerland
(34) Urbanski, T. (1965) *Chemistry and Technology of Explosives,* Vol. 2, Pergamon Press, New York, NY, pp. 175–191

(35) Federoff, B. T., & Sheffield, O. E. (1966) *Encyclopedia of Explosives and Related Items,* PATR 2700, Vol. 3, C474-488, Picatinny Arsenal, Dover, NJ

(36) Newhouser, C. R. (no date) *Introduction to Explosives,* International Association of Chiefs of Police, Gaithersburg, MD

(37) *Military Explosives* (1967) TM 9-1300-214 7–75 ff, Departments of the Army and the Air Force, Washington, DC

(38) Federoff, B. T., & Sheffield, O. E. (1960) *Encyclopedia of Explosives and Related Items,* PATR 2700, Vol. 1, A158–165, Picatinny Arsenal, Dover, NJ

(39) Walker, R. F., & Castorina, T. C. (1974) *Chemical Characterization of Energetic Materials, their Decomposition Products and their Residues—Progress in the Identification and Detection of Explosives,* Technical Memorandum 2136, Picatinny Arsenal, Dover, NJ

(40) Coates, A. D., Freeman, E., & Kuhn, L. P. (1970) *Characteristics of Certain Military Explosives,* Report No. 1507, Ballistic Research Laboratories, Aberdeen Proving Ground, MD

(41) Federoff, B. T., & Sheffield, O. E. (1966) *Encyclopedia of Explosives and Related Items,* PATR 2700, Vol. 3, C405–417, Picatinny Arsenal, Dover, NJ

FRUITS AND FRUIT PRODUCTS

Microbiological Assay with *Lactobacillus plantarum* for Detection of Adulteration in Orange Juice

CARL E. VANDERCOOK and DORA C. SMOLENSKY
Fruit and Vegetable Chemistry Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Pasadena, CA 91106

A microbiological assay has been developed to help detect adulteration in orange juice. Under standard assay conditions with diluted orange juice, the growth of *Lactobacillus plantarum* is proportional to the concentration of juice in the assay mixture. Imitation orange beverages did not support growth. Growth was also independent of the normal levels of common beverage ingredients such as sugar, acids, butylated hydroxyanisole, and orange oil. Commercial orange juices reconstituted from concentrates from various sources were assayed by the microbiological procedure, and the variability of results (coefficient of variation 24%) was about the same as or slightly lower than that for many of the other constituents used to estimate adulteration.

There has been continued interest by governmental regulatory agencies, consumer groups, and citrus processors in the problem of detecting adulteration in orange juice beverages. We recently reported on the concept of a microbiological assay for juice content with an unknown bacterium whose growth was proportional to the orange juice content (1). The unknown bacterium, which was tentatively identified as a *Lactobacillus*, had many of the characteristics of *L. plantarum*. For an assay of orange juice to be generally applicable, we felt it would be best to use a readily available, known organism. The purpose of this investigation was to explore the suitability of *L. plantarum* in this assay and to determine the experimental parameters and variance of the assay.

METHOD

Apparatus and Reagents

(a) *Liquid medium.*—Prepare Bacto lactobacilli MRS broth (Difco 0881-02) according to label. Pipet 10 ml into test tubes, autoclave 15 min at 121°C, and store at 4°C until needed.

(b) *Solid medium.*—Prepare MRS agar from MRS broth by adding 2% agar and bring mixture to boil. While mixture is still hot, pipet 10 ml into test tubes. Sterilize and store the tubes as in (a).

(c) *Saline.*—0.85% NaCl. Pipet 10 and 9 ml aliquots (twice as many 10 as 9 ml aliquots) into test tubes and sterilize as in (a). (Tubes with 10 ml are used for suspending and washing cells for inoculum; tubes with 9 ml are used for 1 + 9 dilution of final inoculum.)

(d) *Test organism.*—*Lactobacillus plantarum*, NRRL B-1921 (Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604).

(e) *Spectrophotometer.*—Cary Model 14 (Varian, Palo Alto, CA).

(f) *Sterile hood.*—White-Roomette (Ultrasonic Industries, Plainview, NY).

(g) *Incubator.*—18″ sq box equipped with blower fan and thermoregulator, ±0.1°C (Lab Apparatus Co., Cleveland, OH).

(h) *High-speed centrifuge.*—Sorval Model RC-5 (Du Pont Instruments, Newtown, CT).

(i) *Low-speed centrifuge.*—International Model HN (International Equipment, Needham Heights, MA).

Stock Culture

Grow *L. plantarum* in MRS broth, and maintain as stab culture on MRS agar. Store stab cultures at 4°C, and subculture monthly.

Inoculum

For preparation of inoculum for assays, inoculate MRS broth from stab culture, and incubate broth at 37°C overnight (17–24 hr). Centrifuge cells at low speed (1000 × *g*) under aseptic conditions, and discard supernate. Wash the cells once with 10 ml saline, centrifuge, and discard wash. Suspend cells in 10 ml saline, and dilute suspension 1 + 9 with additional saline to give inoculum. Use 1 drop of inoculum to inoculate each assay tube.

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

Received April 1, 1976.

Sample Preparation and Assay

Centrifuge single strength juice or concentrate diluted to single strength at 17,000 × *g* (or greater if necessary) for 15 min to give clear sample. Measure per cent soluble solids (sucrose scale of refractometer) of clarified juice. Adjust pH of 20 ml of clarified juice to 6.5 with NaOH and dilute to 100 ml. Prepare 5 replicate culture tubes (25 × 150 mm) with 10 ml of each assay mixture, and autoclave 15 min at 121°C. Hold 2 tubes as uninoculated controls, and inoculate 3 tubes with organism. Incubate all tubes 30 hr at 37°C. Determine bacterial growth (as turbidity) by measuring absorbance in 1 cm cells of the inoculated assay mixture against uninoculated control at 550 nm. Express results as: Microbiological assay value = (absorbance × 11.8)/% soluble solids.

Results and Discussion

Various aspects of the experimental procedure were investigated to determine their influence on the overall experimental error. The inoculum concentration, time of sterilization, and time and temperature of incubation were considered individually.

We found (1) on a similar *Lactobacillus* species that inoculum dilutions greater than 10-fold (i.e., 100-, 1000-, 10,000-fold) required an excessive time for the bacterium to reach its maximum growth, even though the values for final growth were about equal. A 10-fold dilution of the cell suspension was selected for the assay because of cell settling problems associated with the higher concentration. In nutrient broth, however, inoculation with the straight cell suspension produced slightly higher growth than inoculation with a 10-fold dilution of the suspension (Table 1). The effect of small changes in inoculum concentrations on cell growth was evaluated on an orange juice sample by inoculating the assay mixture with different dilutions. Table 1 shows the absorbance of assays inoculated with different dilutions (1+4, 1+7, 1+9, 1+11, and 1+19) of the cell suspension. Growth in the assay did not differ significantly ($P < 0.05$) over the range of inoculum tested. For a check on the day-to-day variations in inoculum concentration, the absorbance of the 1 + 9 dilution was measured after the inoculation step. The range of inoculum absorbance at 550 nm, measured on 11 different days, was 0.39–0.70 (mean and standard deviation 0.50± 0.09). This range is less than that of the inoculum dilutions which produced no significant difference in the assays in Table 1.

Table 1. Effect of inoculum concentration on growth (measured as absorbance) of *L. plantarum*

Sample[a]	Inoculum diln	Inoculum absorbance	Mean absorbance
A	undild[b]	—	0.900
A	1 + 9	—	0.840
B	1 + 4	0.77	0.385[c]
B	1 + 7	0.51	0.393[c]
B	1 + 9	0.41	0.392[c]
B	1 + 11	0.35	0.386[c]
B	1 + 19	0.21	0.394[c]

[a] A = nutrient broth, B = orange juice.
[b] Cells suspended in 10 ml saline.
[c] Mean of 7 replicates for each dilution. Analysis of variance showed that dilution did not significantly affect absorbance (calculated $F = 1.60$; $F_{0.05} = 2.69$).

The effects of inoculum times (17, 24, and 48 hr) were tested on inoculum concentration and on the final bacterial growth in the assay mixture. Absorbances for the inocula from the 3 incubation periods were within the 0.39–0.70 range reported above. Absorbance did not differ greatly between the assays inoculated with the 17- or 24-hr incubated inoculum (means 0.339 and 0.336, respectively), but the 48-hr incubated inoculum produced a 13% lower growth in the assay.

The effect of sterilization time was determined by autoclaving the same sample preparation for 12, 15, and 20 min. The mean absorbance values were 0.389, 0.380, and 0.368, respectively. Apparently small deviations from the standard 15 min would not cause any problems.

The optimum temperature for growth of *L. plantarum* was reported as 30–35°C (2). In the orange juice system, however, growth was maximum at 34–37°C (Table 2). Small fluctuations between 34 and 37°C should not affect the analyses.

Growth of the bacterium in the assay medium was plotted as a function of time for 72 hr (Fig. 1). We chose 30 hr, when growth was 95% of the maximum, for convenience. Samples inoculated by 10 o'clock in the morning can be read at 4 o'clock the next afternoon.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was accepted by the Association. *See* (1976) *JAOAC* **59**, 389.

This report of the Associate Referee, C. E. Vandercook, was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Table 2. Effect of incubation temperature on growth (measured as absorbance) of *L. plantarum* in orange juices

Orange juice	Temp., °C	Absorbance	Mean
A	30	0.300, 0.290, 0.305, 0.310, 0.305, 0.310, 0.300, 0.310, 0.310, 0.295	0.303
A	37	0.350, 0.330, 0.350, 0.360, 0.350, 0.360, 0.350, 0.340, 0.330, 0.360	0.348
B	34	0.316, 0.318, 0.316, 0.316, 0.320	0.317
B	37	0.318, 0.316, 0.320, 0.316, 0.316	0.317
B	40	0.290, 0.295, 0.299, 0.286, 0.290	0.292

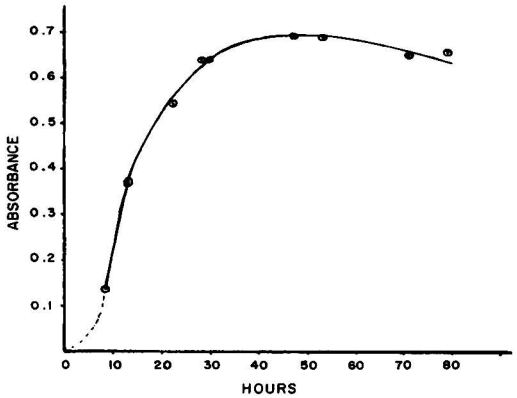


FIG. 1—Growth of *L. plantarum* in orange juice assay as a function of time.

The choice of a 20% juice level in the assay mixture was based on several considerations including convenience of sample handling and final absorbance values. However, the primary reason is that, under the current experimental conditions, bacterial growth, as a function of juice content, is in the middle of the linear response range at the 20% juice level. The linear response range was checked for 3 representative orange juice samples. The standard assay procedure was followed except for the juice aliquots which were 5, 10, 20, 50, and 75 ml per 100 ml final volume. The response was linear up to 50% juice in the assay mixture ($r = 0.9985, 0.9956, 0.9995$). With more than 50% juice in the assay mixture, bacterial growth was inhibited.

For testing the agreement between experiments, we prepared a large batch of composite orange juice for assay as in the standard procedure except for proportionally larger volumes. Culture tubes with 10 ml assay mixture were frozen until needed. A set of these tubes was withdrawn for inclusion in each group of determinations. Table 3 shows the results, from the composite orange juice, of assays by 2 analysts on 9 days. As might be expected, the variance within a single day was significantly lower than the variance from day to day. From the analysis of variance (3), the standard deviation due to experimental error was ±0.008 or 2.4% of the mean. The standard deviation between determinations on different days was ±0.018 or 5.4% of the mean, which might be expected on a day-to-day basis.

Table 3. Replication of assay for composite orange juice by 2 analysts on 9 days, with analysis of variance

Analyst	Measured absorbance[a]	Daily means	Analyst means
A	0.337, 0.342, 0.346, 0.333, 0.337 0.325, 0.329	0.336	
A	0.333, 0.333, 0.329, 0.329, 0.329	0.331	
A	0.325, 0.316, 0.320	0.320	0.331
B	0.340, 0.340, 0.370	0.350	
B	0.340, 0.340, 0.325	0.335	
B	0.330, 0.330, 0.330	0.330	
B	0.310, 0.310, 0.320	0.313	
B	0.340, 0.330, 0.330	0.333	
B	0.340, 0.320, 0.340	0.333	0.333
Overall composite orange juice mean and standard deviation			0.332±0.011

Source of variance	Degrees of freedom	Sums of squares ($\times 10^6$)	Mean squares ($\times 10^6$)
Determinations	8	2572.5	321.6
Error	24	1525.6	63.6
Total	32	4098.1	
	$F = 5.06$	$F_{0.01} = 3.36$	

[a] Not corrected for soluble solids.

Table 4. Effect of beverage ingredients on growth (measured as absorbance) of *L. plantarum* in orange juices

Orange juice	Ingredient added	Level[a]	Absorbance[b]
A	none	—	0.393,[d] 0.398[d]
A	BHA[c]	2 ppm	0.398,[d] 0.405[d]
A	orange oil	30 ppm	0.403[d]
A	orange oil	90 ppm	0.401[d]
B	none	11.8% soluble solids	0.336[d]
B	sucrose	13.0% soluble solids	0.340[e]
B	sucrose	13.1% soluble solids	0.340[e]
B	sucrose	14.4% soluble solids	0.327[e]
B	sucrose	15.2% soluble solids	0.327[e]
B	sucrose	16.7% soluble solids	0.334[e]
B	sucrose	17.5% soluble solids	0.329[e]
B	sucrose	19.5% soluble solids	0.318[e]
B	sucrose	21.9% soluble solids	0.301[e]
B	sucrose	39.7% soluble solids	0.258[e]

[a] Based on equivalent single strength orange juice. The level in the assay mixture is 1/5 that of the single strength juice.
[b] Measured absorbance; not corrected to 11.8% soluble solids.
[c] Butylated hydroxyanisole.
[d] Mean of 2-4 replicates.
[e] Single sample.

Orange juice-based beverages often contain orange oil for flavoring, butylated hydroxyanisole (BHA) as an antioxidant, and sugar. The effects of these ingredients are reported in Table 4. Orange oil and BHA, at the low levels normally occurring in beverages, did not affect the growth of the bacterium, but growth of *L. plantarum* was inhibited by high sugar concentrations. This should not present a problem when assaying single strength orange juices with a normal range of sugar concentrations (10–15%), since the standard dilution (20%) would yield a 2–3% level of sugars in the assay mixture. When the sugar concentration in the assay mixture exceeded 4–5%, growth was inhibited. As noted above, the bacterial growth deviated from linearity when the juice content in the assay mixture exceeded 50%. At the 75% juice level (7.5–11.3% sugar), growth was reduced to 92% of the projected linear growth. The effect of added citric acid on the assay was tested on an orange juice with acidity levels adjusted to 2.24 and 2.92 (calculated as per cent anhydrous citric acid). There was no significant difference in the final mean growth (0.307 and 0.305, respectively). Two brands of a synthetic breakfast orange-flavored drink were prepared according to the label and assayed by the standard method. There was no measurable growth of the bacterium in either sample. It should be mentioned in passing that *L. plantarum* is not specific for orange juice. It also grows in lemon and grapefruit juices as well as some non-citrus juices under the assay conditions (1).

Growth-limiting factor(s) in orange juice have not been identified. However, since *L. plantarum* is used in assays of both nicotinic acid and pantothenic acid (4), the effects of these and other vitamins added to the assay mixture were investigated. A commercial multi-vitamin tablet plus folic acid was dissolved in water and an aliquot was added to the assay mixture before dilution to final volume. The amounts of the added vitamins in the final assay mixture were as follows: vitamin A (acetate), 50 USP units; vitamin D (ergosterol), 4 USP units; ascorbic acid, 0.5 mg; thiamine, 0.02 mg; riboflavin, 0.025 mg; pyridoxine, 0.01 mg; niacinamide, 0.2 mg; pantothenic acid, 0.01 mg; cyanocobalamin, 0.01 μg; and folic acid, 5 μg. These vitamin levels amounted to a substantial increase over those naturally present in the orange juice aliquot; however, there was no significant change in the growth of *L. plantarum* in the fortified juice over the control. Therefore, these vitamins are apparently not the growth-limiting factors in orange juice.

Orange juices and orange concentrates (reconstituted) from Florida, California, and Mexico were assayed by this procedure. Data for these samples are presented in Table 5 as absorbance values. Each value should be considered as a parameter of the particular sample. The mean and standard deviation for the 30 samples are

Table 5. Microbiological assay values for 30 orange juices from 3 regions

Source	Sample absorbance means[a]	Source mean
California	0.456, 0.364, 0.616, 0.425, 0.411, 0.464, 0.271, 0.341, 0.413, 0.387, 0.490, 0.488	0.410
Florida	0.318, 0.314, 0.370, 0.400, 0.384, 0.350, 0.295, 0.268, 0.309, 0.253, 0.205, 0.425, 0.460	0.335
Mexico	0.355, 0.535, 0.352, 0.234, 0.385	0.372
Mean	0.378	
Std dev.	0.092	
Coeff. of var.	24%	

[a] Absorbance adjusted to 11.8% soluble solids (Brix).

0.378±0.092. The standard deviation is 24% of the mean which is lower than standard deviations for some of the juice constituents investigated previously in this laboratory (5).

Absorbance would be lower for an adulterated than for a full strength orange juice, and if the value decreased below a predetermined level the product would be rejected. Assays of several samples from a given source would increase the probability that authenticity would be correctly identified. Nevertheless, because of the variability between samples, the microbiological assay probably would not be used as the only test of authenticity. Numerous workers (6–10) have shown that data with relatively high coefficients of variation can be processed by use of certain statistical combinations to produce results with significantly lower variance. It will be necessary to analyze more samples by this assay and other procedures (5) to select the best combination of parameters. It is also possible that organisms with different nutrient requirements might complement each other in a series of assay procedures for detecting adulterations. It is recommended that study be continued.

References

(1) Vandercook, C. E., Smolensky, D. C., & Nakamura, L. K. (1976) *J. Food Sci.* **41**, 709–710

(2) Buchanan, R. E., & Gibbons, N. E. (1975) *Bergey's Manual of Determinative Bacteriology*, 8th Ed., The Williams & Wilkins Co., Baltimore, MD, p. 585

(3) Snedecor, G. W. (1956) *Statistical Methods*, 5th Ed., The Iowa State University Press, Ames, IA, p. 271

(4) *Official Methods of Analysis* (1970) 11th Ed., AOAC, Washington, DC, sec. 39.079

(5) Vandercook, C. E., Price, R. L., & Harrington, C. A. (1975) *JAOAC* **58**, 482–487

(6) Steiner, E. H. (1949) *Analyst*, **74**, 429–438

(7) Rolle, L. A., & Vandercook, C. E. (1963) *JAOAC* **46**, 362–365

(8) Lifshitz, A., Stepak, Y., & Brown, M. (1971) *JAOAC* **54**, 1266–1269

(9) Lifshitz, A., Stepak, Y., & Brown, M. B. (1974) *JAOAC* **57**, 1169–1175

(10) Vandercook, C. E., Mackey, B. E., & Price, R. L. (1973) *J. Agric. Food Chem.* **21**, 681–683

ALCOHOLIC BEVERAGES

Forms of Iron in Beer

SHELA GORINSTEIN
Department of Pharmaceutical Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

Different forms of iron (total, ionic, and complexed) were investigated by colorimetry, emission spectroscopy, spectrophotometry, and electrophoresis on agar gel with the following results: The lowest concentration of complexed iron occurs in the sample of highest colloid-protein stability (limit of precipitation); the colloid protein stability is directly dependent on the content of complexed iron in hordein-globulin fractions; a decrease in hydrogen ion concentration leads to an increase in the content of complexed iron; a low redox potential corresponds to high protein stability; the change in fractional protein composition depends on the amount of complexed iron; the content of proteins in salt and in alcoholic fractions increases with added ferric ions, resulting in decreased colloid protein stability.

Iron is one of the important trace elements in the mineral composition of beer (1–3) because it is physiologically active, it influences the quality of the beer, and it affects nitrogen exchange in it (4–15). Iron in small quantities can also be a catalyst in redox reactions. Therefore, the stability of beer depends on the content of iron-reactive polyphenols (16), melanoidins, reductones (17), tanninogens (18), proteins, and dissolved oxygen (4–7, 19–21).

Trace metals in beer result both from natural sources and arise during processing. Iron may appear in the cationic Fe(III) or Fe(II) form or in an anionic complex, also with a valence of +3 or +2. There may also be organic compounds in beer which do not undergo dissociation but which contain iron. The metals which are tied up in complexes are not highly active. The mobility of the metal ions depends on the stability of the complexes, which, in turn, is determined by the nature of the complex, the pH of the solution, and the redox potential. Therefore, the complexed metal ions can serve as a reservoir of free ions; a change in the medium releases the ions, which can then form new complexes. The divalent and trivalent iron ions are in equilibrium. The equilibrium may shift in the direction of the divalent ion in the presence of natural reducing agents. The trivalent iron ions can result from the oxidation of Fe(II) with atmospheric oxygen or on reduction of Cu(II) to Cu(I). The presence of copper ions thus affects the stability of the medium. The turbidity of the medium may also be determined by the quantity and valence of the cationic form of iron, since Fe(III) can form insoluble complexes with various components of the ethanolic medium (22, 23). Therefore, a knowledge of the total iron ion composition of the medium is not enough to determine the stability; it is necessary to know those forms which the iron takes in the medium (24). Thus the different forms of iron, the dynamic changes of redox potential in the investigated samples, the effect of these changes on the forms of iron, and the effect of the iron on the colloidal stability of beer were investigated. Techniques available for the analysis of iron content are spectroscopy (25–28), atomic absorption spectrometry (29–31), and colorimetry (32).

Experimental

The investigation was carried out on Zhuguli nonfiltered beer (Kolos Breweries, Lvov, U.S.S.R.) produced by the double-decoction method from 60% light malt and 40% nonmalted adjuncts. Standards of comparison were brews clarified by cotton filtering masses: Kineshma (control) and Evlakh[1] (test). Kineshma was selected as the control because it was processed by a more standard procedure. The 2 are distinguished by their filter-

Received September 26, 1975.

[1] Kineshma and Evlakh are the Russian names of samples of cotton fibers. Kineshma mass is 34 nephelos units and Ev'akh mass is 55 nephelos units, one differing from the other in filtering ability.

ing ability, which is superior for Evlakh mass. The investigation is based on 30 experiments.

Total Iron

The total iron content in alcoholic beverages was determined by emission spectroscopy. Fifty ml samples were dried and ashed, the ashes were mixed with standards and a buffer, and the resulting mixtures were burnt in an ac electric arc on a Spectrograph I JSP-28. Iron was also determined by the method of Vondenhof and Beindorf (33). The quantitative determination of iron in the presence of the other elements is based on the formation of complexes with PAN (a polyamide synthetic fiber) (2).

In order to determine total iron content the sample was heated to 480°C, treated with HNO_3, and dissolved in 18% HCl. The solution was transferred in portions to a separatory funnel and the iron as $FeCl_3$ was extracted into ether. The extracts were combined and the solvent was removed. The product was then dissolved in HCl and H_2SO_4 was added. The volume was measured and the spectrophotometric determination of total iron present was made at 520 μm (2, 22, 34).

Cationic Form of Iron

The cationic form of iron was determined by chromatography on an ion exchange column of cationic resin KY-2 in the potassium form. The column was eluted with 20% KCl and the Fe(III) + Fe(II) in the eluate was determined colorimetrically by adding *o*-phenanthrolene or α,α'-dipyridyl in the presence of hydroxyamine.

Complexed Iron

The amount of complexed iron was determined by the difference between total and cationic iron. The complexed form of iron was also determined by concentration and fractionation of the proteins in beer according to the tannin-caffeine method (35). The sample of beer (400 ml) was treated with 30 ml 4% tannin solution and the precipitate was dissolved in twice its weight of caffeine. Samples of the concentrated protein (0.04 ml) were subjected to horizontal electrophoresis for 5 hr at 40–80 v on EFA-1, a veronal-medinal pH 8.6 buffer containing 1.5–2.0% agar gel. After the fractions were separated by electrophoresis, complexed iron was determined in each fraction by emission spectroscopy and spectrophotometry.

Proteins were also concentrated and fractionated by the micro-chromatographic method, which uses columns of Sephadex G25, molselect G25, Acrylex P30, kieselgel Stabifix, kieselguhr, bentonite, polyacrylamide, polyamide resin Perlon, silica gel, silicic acid, aluminum oxide, Florisil, and Perlite. The ratio of adsorbent to sample was 50:1. Columns were eluted with 0.05*N* NaCl and dialysis was performed in glucose solution for 48 hr. The concentrated protein was fractionated by horizontal electrophoresis on agar gel.

To determine the complexed and ionic forms of iron we also used another method of fractionation based on the solubility of proteins in different solutions. Proteins were extracted successively from the beer samples with solutions of 5% potassium sulfate, 0.2% NaOH, and 70% ethanol. The extracts were filtered and the proteins were precipitated by adding 10% trichloroacetic acid to each filtrate. The filtrates remaining after precipitation were also investigated. Iron in the sediments and the washing was determined spectroscopically.

Other Parameters

Foam stability, colloid protein and biological stability, redox potential, and pH and rH were determined by conventional methods. Common nitrogen was determined by the micromethod of Dumas and Kjeldahl, and a factor of 6.25 was used to convert the nitrogen value to crude protein. The colloidal stability of beer was determined by using the limit of precipitation of ammonium sulfate. As the limit of precipitation decreased, the colloidal stability increased.

To calculate the redox potential, EH, we used a method based on the measurement of the electric potential as determined by the pH and the voltage:

$$rH = \frac{2\ EH}{0.1984\ T} + 2\ pH$$

where $rH = \log[H_2]$; $pH = -\log[H^+]$; EH = redox potential of the system; and T = absolute temperature. The potential of a 50 ml sample was determined with a platinum-calomel electrode system.

In a series of experiments, standard solutions of the cations of bi- and trivalent iron (Fe^{+2} with CNS^- [pK 0.07]; Fe^{+3} with CNS^- [pK 2.3]; Fe^{+2} with CN^- [pK 24]; and Fe^{+3} with CN^- [pK 31]) were added to samples of control beer. Results (Fig. 1) show the dependence of colloid protein stability on iron in different forms.

Results and Discussion

Results show that there is a relatively large amount of complexed iron in unfiltered beer (Table 1). Most of the iron in the test sample was in cationic form. Data in Table 1 show that this sample exhibited higher stability by its larger limit of precipitation.

The protein fraction of beer was concentrated

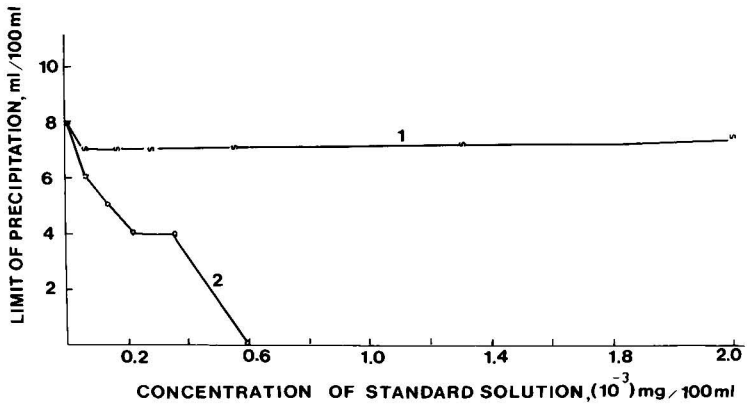


FIG. 1—The colloid protein stability of beer as a function of the content of ionic forms of iron: 1, bivalent iron; 2, trivalent iron.

by chromatography and fractionated by electrophoresis into 2 groups: the albumins-glutelins and the globulins-hordeins. The ability of the different adsorbents to concentrate the protein fractions is as follows (*see* Table 2 and Figs. 2 and 3): aluminum oxide < silica gel < Perlite < Florisil < polyamide resin Perlon < Acrylex P 30 < silicic acid < polyacrylamide < molselect G25 < kieselgel Stabifix < kieselguhr < Sephadex G25. All the adsorbents were added to the beer samples at 20–25°C. The results show that protein concentration is dependent on the different adsorbents (the change of total protein, β-globulin, and iron is in a definite order).

The complexed and ionic forms of iron were also determined in the different beer fractions (Table 3). The bond strengths between the complexed iron and proteins were determined by the ratio of the quantity of complexed iron in protein fractions to the total amount in beer. The data obtained show that iron is found in complexes with proteins of all fractions, and that iron present in the sediments is in complexed form and in the filtrates in free ionic form.

Table 1. Iron content (10^{-3} mg/L) of treated and untreated beer samples

Form of iron	Non-filtered	Control	Test
Total (spectroscopy)	170.15	118.34	54.17
Cationic, Fe(II) + Fe(III) (colorimetry)	29.77	22.20	30.00
Complexed (difference)	140.38	96.14	24.17
Limit of precipitation, (ml $(NH_4)_2SO_4$/100 ml)	—	8.0	11.0

Beside the determinations of the total, ionic, and complexed iron forms, we determined the effect of changes in the pH of the medium on the relative amounts of each form present. The pH of the original products was 4.5. A series of samples were prepared and the pH was altered from 0 to 7 by adding sulfuric acid or sodium hydroxide. After one week, equilibrium between the different forms of iron was attained in the samples. The pH of each sample was rechecked and the total, cationic, and complexed iron contents were determined. The nature of the variation in percentage of complexed iron with respect to changes in pH is almost identical in each of the 3 samples (nonfiltered, control, and test). The differences that do exist can be explained by the presence of different amounts of complex-forming compounds in each of the samples and by differences in their ability to complex with iron. Our experiment shows that with an increase in concentration of hydrogen ions the percentage of complexed iron decreases.

The results of the calculations of the redox potential (EH) are given in Table 4. The data show that the lowest redox potential is associated with the highest protein stability. Redox ability is determined by the presence of reducing agents which protect beer from oxidation. These reducing agents are melanoidins, polyphenols, proteins, carbohydrates, sulfoxides, and ascorbic acid. All of these substances possess reducing

Table 2. Complexed iron (1×10^{-3} mg/L) removed by different adsorbents

Index	Electrophoresis			Chromatography and electrophoresis													
	Non-filtered	Control	Test	Bentonite	Polyacrylamide	Polyamide resin Perlon	Acrylex P30	Molselect G25	Sephadex G25	Kieselgel Stabifix	Kieselguhr	Silica gel	Silicic acid	Aluminum oxide	Florisil	Perlite
Iron	140.38	96.14	24.17	114.16	118.17	130.14	125.18	109.21	101.05	104.25	120.16	137.77	123.18	139.14	134.27	137.12
Iron in albumin + glutelin	100.19	70.00	18.34	81.93	85.38	95.89	91.74	78.03	71.04	73.31	90.02	99.61	90.04	99.96	96.53	98.98
Iron in hordein + globulin	40.19	26.14	5.83	32.23	32.79	34.25	33.44	31.18	30.01	30.94	30.14	38.16	33.14	39.18	37.74	38.14
Limit of precipitation, ml $(NH_4)_2SO_4$/100 ml	—	8.0	11.0	7.5	6.5	6.0	6.0	7.0	7.5	7.0	7.5	5.0	6.5	4.0	5.5	5.0

ability and therefore establish a certain redox potential. The oxidized form, Fe(III), as opposed to Fe(II), decreases the effectiveness of the melanoidins. The greater the concentration of Fe(III), the higher the redox potential of the system, according to the Nernst equation.

The table also presents the results of adding the ionic forms of Fe(III) and Fe(II) to the control sample. The main quantitative indices of beer (turbidity, foam stability, colloid protein, and biological stability) are most influenced by the introduction of the oxidized form of iron.

Beer stability also depends on the concentration of those forms of iron capable of complexing with the different protein fractions. Adding bivalent iron did not greatly affect the colloid protein stability of beer, while trivalent iron added at levels of $3.8–7.0 \times 10^{-3}$ mg/100 ml sharply decreased it. This confirms the supposition that free Fe(II) ions are less active than Fe(III). These changes also depend on whether the Fe(II) and Fe(III) are added as a complex with CNS^- or CN^-. There is little change in the latter case, as would be expected from the pK values (Fig. 1).

It is interesting to compare the results of Tables 3 and 5 obtained by investigation of different protein fractions. From Table 5, we see that the protein composition in the sample affects the mineral composition, especially that of iron. In the nonfiltered sample without added Fe(III), there was more protein in the salt fraction compared with the test and control samples. After Fe(III) was added, the fractional composition of the protein changed; the greatest concentration was in the salt and alcoholic fractions and the lowest concentration was in

Table 3. Free ionic and complexed forms of iron ($\times 10^{-3}$ mg/L)

Index	Non-filtered	Control	Test
Filtrates:			
Aqueous (albumin)	16.22	3.14	2.01
Salt (globulin)	10.18	7.14	4.18
Alkaline (glutelin)	3.08	2.46	1.12
Alcoholic (pliolamine + hordein)	14.29	12.49	7.34
Sediments:			
Aqueous	53.09	41.15	9.31
Salt	30.14	20.14	2.09
Alkaline	57.10	27.16	10.03
Alcoholic	10.05	9.14	4.11

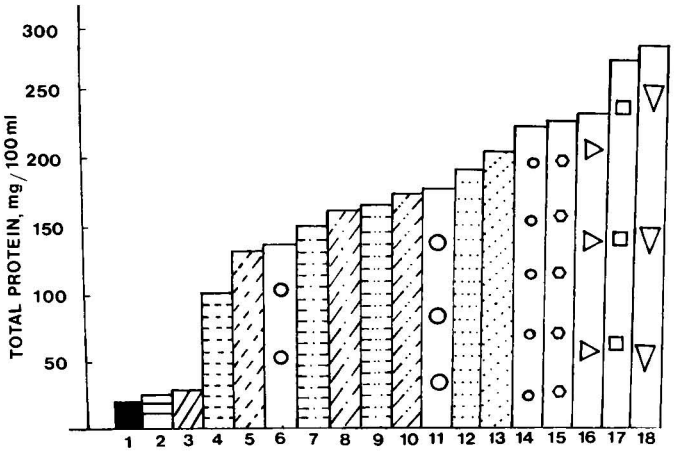


FIG. 2—Total protein concentration obtained after chromatography on various adsorbents: 1, Sephadex G25; 2, Sephadex G100; 3, Sephadex G50; 4, aluminum oxide; 5, silica gel; 6, Perlite; 7, Florisil; 8, Perlon; 9, Acrylex P30; 10, silicic acid; 11, polyacrylamide; 12, Molecular Sieve; 13, Sephadex G75; 14, kieselguhr; 15, bentonite; 16, ammonium sulfate; 17, tannin-caffeine; 18, kieselgel Stabifix.

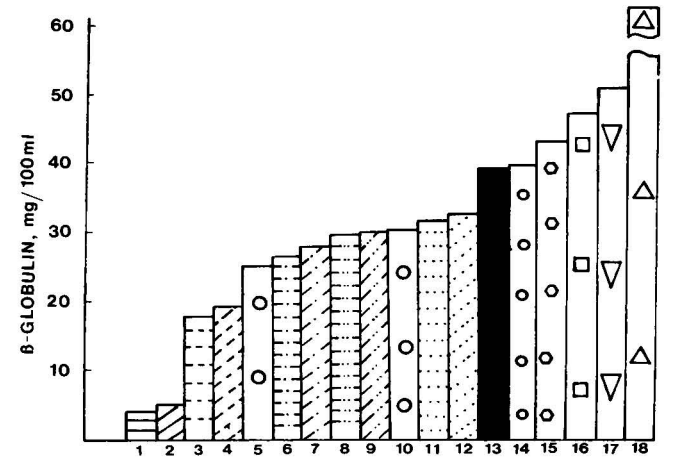


FIG. 3—β-Globulin concentration obtained after chromatography on various adsorbents: 1, Sephadex G75; 2, Sephadex G25; 3, Sephadex G100; 4, Sephadex G50; 5, aluminum oxide; 6, silica gel; 7, Perlite; 8, Florisil; 9, Perlon; 10, Acrylex; 11, silicic acid; 12, polyacrylamide; 13, Molecular Sieve; 14, kieselguhr; 15, bentonite; 16, kieselgel Stabifix; 17, ammonium sulfate; 18, tannin-caffeine.

the soluble aqueous fraction. Therefore, we can conclude that the role of iron is important, and the fractional composition is dependent on the iron content. The presence of iron also resulted in an increase in the salt and alcoholic fractions. Aqueous and alkaline fractions were not significantly affected.

Acknowledgments

The author is grateful to S. Sarel (The Hebrew University of Jerusalem) for his interest in the work. The author also wishes to express appreciation to R. Victor for her helpful discussions and comments and also to M. Fine (The Hebrew University of Jerusalem).

Table 4. Effect of adding iron on the physicochemical indices of beer

Index	Sample			Control with added iron cations, 4.5×10^{-3} mg/100 ml	
	Nonfiltered	Test	Control	Fe(III)	Fe(II)
pH	4.75	5.20	5.19	5.02	5.00
EH	−106.2	−78.2	−89.2	−120.5	−90.2
rH	14.5	12.5	13.8	14.68	13.5
Height of foam, mm	—	—	20.0	5.0	13.0
Limit of precipitation, ml $(NH_4)_2SO_4$/100 ml	—	9.0	8.0	4.0	7.0
Biological stability, days	—	9.0	8.0	1.0	4.0
Foam stability, min	—	—	2.0	0.3	1.4

Table 5. Dependence of iron changes on fractional protein composition

Sample	Total amt protein, mg/100 ml	Protein fractions			
		Aqueous	Salt	Alcoholic	Alkaline
Nonfiltered	358.75	38.27	183.40	102.41	34.67
Control	341.88	28.34	179.25	100.29	34.00
Test	297.50	27.23	157.90	80.37	32.00
Nonfiltered with Fe(III)	358.75	20.00	200.34	118.44	19.97
Control with Fe(III)	341.88	14.93	196.61	113.34	17.00
Test with Fe(III)	297.80	13.92	174.27	89.31	20.00

References

(1) Ault, R. (1970) *J. Inst. Brew. London* **76**, 299–301

(2) Gorinstein, S., Vizgert, R., & Kiseleva, L. (1971) *NTI Tsintipishcheprom* **5**, 7–9

(3) Postel, W., Drawert, F., & Quevenc, U. (1972) *Brauwissenschaft* **25**(11), 341–346

(4) Gramshaw, J. W. (1967) *J. Inst. Brew. London* **73**, 258–270

(5) Gramshaw, J. W. (1967) *J. Inst. Brew. London* **73**, 455–472

(6) Gramshaw, J. W. (1968) *J. Inst. Brew. London* **74**, 20–38

(7) Gramshaw, J. W. (1969) *J. Inst. Brew. London* **75**, 61–83

(8) Barrett, J., Griffiths, C. M., & Kirsop, B. H. (1967) *J. Inst. Brew. London* **73**, 445–450

(9) MacWilliam, I. C. (1968) *J. Inst. Brew. London* **74**, 38–54

(10) Ayrapaa, T. (1968) *J. Inst. Brew. London* **74**, 169–178

(11) Engan, S. (1970) *J. Inst. Brew. London* **76**, 254–261

(12) Mantschev, S., & Dechatov, G. (1970) *Brauwissenschaft* **23**(2), 56–61

(13) Venskyavichus, J., Gorinstein, S., & Makshtyalene, Z. (1971) *Sadovod. Vinograd. Vinodel. Mold.* **26**(4), 33–36; thru *Chem. Abstr.* (1972) **76**, 23802*w*

(14) Gorinstein, S. (1973) *J. Food Sci.* **38**, 1264–1266

(15) Gorinstein, S. (1975) *J. Agric. Food Chem.* **23**, 45–47

(16) Jacobsen, T., Lie Sturla, & Chapon, L. (1974) *Tech. Q. Master Brew. Assoc. Am.* **11**, 225–261

(17) Chapon, L., & Louis, C. (1974) *Biosphere* **5**, 448–456

(18) Dadic, M., Van Gheluwe, J. E. A., & Valyi, Z. (1974) *J. Inst. Brew. London* **80**, 558–564

(19) Schildbach, R. (1974) *Monatsschr. Brau.* **27** (10/11), 217–241

(20) Mostek, J., & Dufek, J. (1973) *Sb. Vys. Sk. Chem. Technol. Praze Potraviny* **E39**, 63–81

(21) Venskevicius, J., Gorinstein, S. B., & Maksteliene, Z. (1973) *Ziet. TSR Aukst. Mokyklu Mokslo Darb. Chem. Technol.* **15**, 413–418; thru *Chem. Abstr.* (1975) **83**, 26226*s*

(22) Patel, G. B., & Ingledew, W. M. (1973) *J. Inst. Brew. London* **79**, 392–396

(23) Steiner, K. (1973) *Brau Rundsch.* **84**(12), 241–243

(24) Ratushnyi, G. D., & Ratushnaya, S. G. (1970) *Ivz. Vyssh. Uchebn. Zaved. Pishch. Tekhnol.* **1**, 11–16

(25) Bergner, K., & Lang, B. (1970) *Dtsch. Lebensm. Rundsch.* **66**(5), 157–164

(26) Schoeneman, L. R., Dyer, H. R., & Earl, M. E. (1971) *JAOAC* **54**, 1247–1261

(27) Tauner, A. (1967) *Obst. Weinbau* 103(5), 180–184
(28) Wright, F. C., & Riner, J. C. (1972) *JAOAC* 55, 662–664
(29) Caputi, A., & Ueda, M. (1967) *Am. J. Enol. Viticult.* 18 (2), 66–70
(30) Roth, F., & Gilbert, E. (1969) *Milteilungen Rebeund Wein, Weinbau and Fruchtvertung* 19, 11–16
(31) Weiner, J. P., & Taylor, L. (1969) *J. Inst. Brew. London* 75, 195–199
(32) Konrad, N. (1960) *Nahrung* 4, 365–373
(33) Vondenhof, T., & Beindorf, H. (1968) *Monatsschr. Brau.* 21(6), 156–157
(34) Gozylak, L. E., & Transkov, J. J. (1969) *Izv. Vyssh Uchebn. Zaved. Pischch. Tekhnol.* 6, 157–158
(35) Gorinstein, S. (1975) *JAOAC* 58, 793–798

Gas-Liquid Chromatographic Determination of Methyl Anthranilate in Wine

RICHARD R. NELSON, TERRY E. ACREE, CHANG Y. LEE, and ROBERT M. BUTTS
Department of Food Science, New York State Agricultural Experiment Station, Geneva, NY 14456

A method is described for the gas-liquid chromatographic determination of methyl anthranilate in wine. Methyl anthranilate is extracted with Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane), concentrated, and quantitated by comparing the peak area with that of an internal standard (*p*-di-*n*-butoxybenzene). A synthetic wine solution (12% ethanol in water) as well as White Riesling and Concord wines containing various concentrations of added methyl anthranilate were analyzed. Recovery of methyl anthranilate in the range of 0.1–10 mg/L averaged 100.26% with an average relative error of 3.94%. The method is both rapid and reliable.

Methyl anthranilate was first discovered in 1895 by Walbaum in neroli oil obtained by the steam distillation of bitter orange flowers (*Citrus aurantium*) (1). It has since been found in other plant materials including mandarin, lemon, bergamot, jasmine, gardenia, and grapes of the *labrusca* species (2). Erdmann and Erdmann were first to characterize methyl anthranilate (3) and several workers have since studied the distribution and determination of this ester (4–7). In 1928, the AOAC (8) listed a colorimetric and a gravimetric method for determining methyl anthranilate. These are still the methods of choice for analyzing grape juice (9). Other useful methods have also been proposed, including a method specifying electron affinity gas-liquid chromatography (GLC) (10) and a fluorometric method (11). A GLC method for detecting and measuring methyl anthranilate in imitation grape flavors (at 430–26,050 ppm) was previously reported (12). This paper presents a GLC method for determining methyl anthranilate in wine. A selective solvent extraction procedure minimizes interferences from fermentation products including ethanol. The method is rapid and sensitive. In addition, it is superior to the various juice determinations cited above for the analysis of wine.

Received April 5, 1976.

METHOD

Reagents and Apparatus

(a) *Freon 113.* — 1,1,2-Trichloro-1,2,2-trifluoroethane (Precision Cleaning Agent, E. I. du Pont de Nemours & Co., from Buffalo Solvent and Chemical Corp., Buffalo, NY). Distill and store in glass.

(b) *Internal standard.* — *p*-Di-*n*-butoxybenzene (No. 586, Chem Service, Media, PA), 250 ppm in Freon 113.

(c) *Methyl anthranilate standard solution.* — 300 ppm methyl anthranilate (No. 159, Eastman Organic Chemicals, Rochester, NY) in ethanol. Add standard to wine samples at desired levels.

(d) *Rotary evaporator.* — Büchi Rotovapor (Rinco Instrument Co., Inc., Greenville, IL) with 20°C water bath.

(e) *Gas chromatograph.* — Hewlett-Packard Model 5830A equipped with Model 18850A GLC terminal and flame ionization detector and 4 m × 3 mm stainless steel column packed with 5% SP-1000 (Carbowax modified with terephthalic acid, Supelco, Inc.) on 100–120 mesh Chromosorb W. Operating conditions: temperatures (°C)—column 180, injector 250, detector 300; nitrogen carrier gas 23 ml/min.

Procedure

Add desired amount of methyl anthranilate standard to 300 ml aliquot of wine. Stir 5 min, add 300 ml Freon 113, cover, and stir 15 min on magnetic stirrer at medium speed. Transfer mixture to 1000 ml separatory funnel and let stand until phases separate. Draw off 250 ml Freon phase and transfer to 500 ml round-bottom flask. Attach flask to Rotovapor and concentrate solution to ca 10–15 ml. Add concentrate to 25 ml volumetric flask containing 1 ml internal standard solution. Rinse round-bottom flask twice with 2–3 ml Freon and add rinses to volumetric flask. Dilute sample to volume with fresh Freon. Sample, now ready for GLC analysis, contains 10 ppm internal standard and 10-fold concentrate of wine extract containing methyl anthranilate.

Calibrate gas chromatograph with standard Freon solution of 10 ppm methyl anthranilate and 10 ppm *p*-di-*n*-butoxybenzene. Collect information during calibration run and store for use in experimental determinations.

Calculation

Retention times and response factors (calculated as peak area ratios) of methyl anthranilate and internal standard are stored in computing accessory of gas-liquid chromatograph. Retention times for Freon 113, internal standard, and methyl anthranilate are 0.79, 10.83, 12.90 min, respectively. During each experimental run, compare amount of methyl anthranilate with amount of internal standard. Use this ratio and ratio from calibration run to calculate automatically amount of methyl anthranilate (ppm) present in sample.

Results and Discussion

Freon 113 was chosen for this procedure because it will not extract water from the sample. Furthermore, interfering fermentation products such as methanol, ethanol, and some organic acids are not extracted. Freon is easy to handle in the laboratory because of its high density, low toxicity, and nonflammability.

Methyl anthranilate was determined in a simulated wine solution (12% ethanol in water), in White Riesling wine (*Vitis vinifera*), and in Concord wine (*Vitis labrusca*). Concord grapes naturally contain methyl anthranilate, while White Riesling grapes do not (5, 13). Samples were analyzed with added methyl anthranilate ranging from 0.1 to 10.0 ppm. A plot of the log of the amount of methyl anthranilate added vs. the log of the peak area ratio (methyl anthranilate/*p*-di-*n*-butoxybenzene) for the simulated wine solution and the White Riesling wine was linear. The correlation coefficients were 0.9972 and 0.9992, respectively. This indicates that the method is consistently accurate and there is no deviation from the theoretical linearity of the analysis at the extremes. Table 1 shows the average experimental recovery of methyl anthranilate in the simulated wine, the White Riesling, and the Concord samples. The overall average recovery was 100.26%, with an average relative error of 3.94%. It is apparent that the method works equally well in all 3 systems. It is also clear that the Freon extracts virtually all of the methyl anthranilate from the aqueous phase and that the ester is not subsequently lost during concentration, due to its extremely low volatility (bp_{15} 135.5°C) (14).

The samples analyzed in this work were 10-fold concentrations of the original wine, but it is important to note that the method could be easily adapted to more concentrated samples. In this way, the sensitivity of the method could conceivably be increased. The limit of detection under the conditions described is at least 0.1 ppm methyl anthranilate in the original wine.

Table 1. Recovery of methyl anthranilate at various levels from a simulated wine solution, White Riesling wine, and Concord wine

Sample	Added, ppm	Found, ppm	Recd, ppm	Rec.,[a] %
Simulated wine	0.10	0.114	0.114	114.0
	0.50	0.490	0.490	98.0
	1.00	0.958	0.958	95.8
	5.00	4.490	4.490	89.8
	10.00	10.320	10.320	103.2
White Riesling	0.00	0.000	0.000	—
	0.10	0.102	0.102	102.0
	0.30	0.293	0.293	97.7
	1.00	0.993	0.993	99.3
	3.00	2.957	2.957	98.6
	10.00	9.770	9.770	97.7
Concord	0.00	0.973	0.000	—
	0.10	1.078	0.105	105.0
	1.00	1.994	1.021	102.1

[a] Overall average recovery = 100.26%; average relative error = 3.94%.

Since the accuracy of the results depends on the peak area measurements, peaks under the methyl anthranilate or the internal standard peak would yield erroneous results. This possibility was investigated by analyzing several wines, with and without methyl anthranilate, by combined GLC-mass spectrometry. Samples of Concord, Niagara, Catawba, Ives, Delaware, and White Riesling wines were extracted, concentrated, and analyzed on a Varian Series 1400 chromatograph with a column as described above. The GLC portion of the system was interfaced with a Bendix Model 12 time-of-flight mass spectrometer modified with a CVC electronics package which makes the system equivalent to the CVC MA-2. The spectra collected indicated that the peaks corresponding to methyl anthranilate and *p*-di-*n*-butoxybenzene were free of interfering compounds.

The method described is a rapid and reliable means for determining methyl anthranilate in wine. It is useful in wine flavor research because of the importance of methyl anthranilate in the flavor chemistry of many wines produced in the Eastern United States and Canada. It is suggested that the method be subjected to collaborative study.

References

(1) Scott, R. D. (1923) *Ind. Eng. Chem.* **15**, 732–733

(2) *Fenaroli's Handbook of Flavor Ingredients* (1971) T. E. Furia & N. Bellanca (Eds.), Chemical Rubber Co., Cleveland, OH, p. 502

(3) Erdmann, E., & Erdmann, H. (1899) *Ber. Deut. Chem. Gesell.* **32**, 1213–1219

(4) Power, F. B. (1921) *J. Am. Chem. Soc.* **43**, 377–381

(5) Sale, J. W., & Wilson, J. B. (1926) *J. Agric. Res.* **33**, 301–310

(6) Holley, R. W., Stoyla, B., & Holley, A. D. (1955) *Food Res.* **20**, 326–330

(7) Roger, N. F. (1961) *Food Technol.* **15**, 309–314

(8) *JAOAC* (1928) **11**, 46–48

(9) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, secs. 12.024–12.028

(10) Mattick, L. R., Robinson, W. B., Weirs, L. D., & Barry, D. L. (1963) *J. Agric. Food Chem.* **11**, 334–336

(11) Casimir, D. J., Moyer, J. C., & Mattick, L. R. (1976) *JAOAC* **59**, 269–272

(12) Brunelle, R. L., Martin, G. E., & Ohanesian, V. G. (1965) *JAOAC* **48**, 341–343

(13) Van Wyk, C. J., Webb, A. D., & Kepner, R. E. (1967) *J. Food Sci.* **32**, 660–674

(14) *The Merck Index* (1968) Merck & Co., Inc., Rahway, NJ, p. 681

Gas Chromatographic Determination of Micro Amounts of Cyanide Residues in Wines, Distilled Liquors, and Other Alcoholic Beverages

BRIAN L. BATES and DONALD R. BUICK
Australian Government Analytical Laboratories, Department of Science, 344 Tapleys Hill Rd, Seaton, South Australia 5023

A gas chromatographic method for the determination of cyanide residues in alcoholic beverages has been developed from procedures previously reported for application to water samples. Quantitatively isolating HCN from alcoholic beverages presented difficulties not encountered with aqueous solutions, particularly in the presence of SO_2 in the sample. HCN was liberated from the acidified sample by heating at 70°C, flushed into an NaOH absorber solution, converted to cyanogen bromide (CNBr), extracted into diisopropyl ether, chromatographed on a Porapak Q column, and detected by an electron capture detector. SO_2 that is present in most wines interfered with the bromination step and caused low recoveries. This interference was eliminated by initially converting any cyanide present in the sample to the stable mercuric cyanide salt and then purging the acidified sample solution of all SO_2. The $Hg(CN)_2$ present was then dissociated by adding KI and the analysis proceeded as previously described. Mean recoveries of 80–97% were obtained for 2–20 μg cyanide from replicate analyses of spiked samples of distilled liquors free of SO_2. The relative standard deviations ranged from 6.1 to 11.1%. Mean recoveries of 65 to 91% were obtained in the same range of cyanide from replicate analyses of spiked wine samples known to contain SO_2, with relative standard deviations ranging from 0.8 to 10.2%. The limit of detection was 0.2 μg HCN.

The analytical investigations described below were undertaken as a continuation of an Associate Referee study to supplement the official AOAC method (1–3) for the qualitative detection of HCN in alcoholic beverages with a quantitative method of appropriate sensitivity, accuracy, and precision. The study was considered in 2 parts. First, we determined the optimum procedure for quantitatively isolating the analyte in as pure a form as possible from samples containing significant quantities of alcohol, other volatile organic compounds, and SO_2, using apparatus that is readily available. Second, we investigated the optimum determinative step, which was limited to comparing a standard colorimetric method of long standing with the somewhat newer technique of measuring HCN in the form of cyanogen bromide by gas chromatography (GC). Such a procedure would be suitable for general use in most regulatory and quality control laboratories.

Experimental

Initially, HCN was isolated from acidic aqueous solutions spiked with potassium ferrocyanide, using Conway microdiffusion cells, absorbed in 0.1*N* NaOH solution (4, 5) over 24 hr, brominated, extracted into diisopropyl ether (6), and detected by gas-liquid chromatography (GLC) on a column of 7% Hallcomid M18 on Gas-Chrom Q with an electron capture detector (7). A calibration curve was linear for 0.1 to 5 μg CN^-. However, poor results were obtained for the same procedure applied to alcohol-water (1+1) spiked with 1–40 μg CN^- as potassium ferrocyanide. The high concentration of ethanol prevented the transfer of HCN from the acidified alcohol-water solution to the NaOH absorber solution; therefore, Conway cells were abandoned in this study.

Next we attempted to isolate HCN from acidified alcohol-water (1+1) spiked with potassium ferrocyanide by distilling at the boiling point, flushing the liberated HCN through a reflux condenser, and absorbing it in a 0.1*N* NaOH trap. HCN was quantitated by GLC as described above. Erratic results were obtained in the range of 5–30 μg added CN^-. We then discovered that *n*-propanol, a common congener in wines and brandy, had a retention time identical to CNBr and could not be separated by adjusting parameters on the Hallcomid M18 column in use. Some *n*-propanol was present in both the

Received March 22, 1976.

Table 1. Recoveries of CN^- added as KCN to 200 ml alcohol-water (1 + 1) and brandy, using method of isolation of HCN for samples free of SO_2, and gas chromatography

Sample	Added, μg	ppm	No. of tests	Recovery				
				Mean,[a] μg	Range, μg	Std dev.	Rel. std dev., %	Av., %
Alcohol-water	10.0	0.05	6	8.0	6.8– 9.2	0.8	9.9	80
Brandy	2.0	0.01	4	1.8	1.5– 1.9	0.2	11.1	90
Brandy	10.0	0.05	5	9.7	8.9–10.4	0.6	6.1	97
Brandy	20.0	0.1	4	17.5	15.6–19.6	1.8	10.3	88

[a] Outliers were not excluded.

alcohol solution and brandy, and distilled with the HCN; this probably accounted for the erratic recoveries at these low levels.

We investigated the 3-methyl-1-phenyl-5-pyrazolone colorimetric determinative step of Epstein (8), as specified in the APHA standard methods manual (9). Initially, we could not obtain stable colors for a calibration curve from standard aqueous solutions of HCN. However, by adding more bis-pyrazolone, we obtained a linear calibration curve from 0 to 15 μg CN^-. However, KCN added to alcohol-water solutions, isolated by the distillation/gas flushing technique, and determined in 0.1*N* NaOH absorbing solution again gave very erratic recoveries, 10–93% at 10 μg CN^-/200 ml solution (0.05 ppm).

A Fisher-Milligan spiral gas washer specified in the APHA method (9) was not available for these experiments and may have proved more efficient for absorbing HCN. We varied the procedure by heating the distillation flask containing the acidified sample at 70°C and supplying cooled condenser water at $\leqslant$20°C, to reduce the amounts of other volatile constituents of the sample reaching the absorber trap and to minimize decomposition of HCN that may occur at higher temperatures of distillation. In this series, 11 colorimetric recoveries ranged from 69 to 104% for 10 μg CN^- added to 200 ml (0.05 ppm). The mean value was 8.5 μg, standard deviation 1.0, and relative standard deviation 11.2%. These results were considerably less precise than that specified in the APHA method, which has a relative standard deviation of 1.7% for water samples.

Nota and Palombari (6) used a Porapak Q column to separate CNBr for subsequent GC determination by electron capture detector. None of the commonly occurring congeners in wine or brandy interfered with the CNBr peak on this column under the operating parameters used. Alcohol-water solutions were spiked at 10 μg CN^-/200 ml (0.05 ppm). The HCN was isolated and quantitated by the procedures described below, *Isolation of Cyanide from Samples Free of Sulfur Dioxide* and *Gas Chromatography*, respectively. Recoveries from replicate analyses were satisfactory and are given in Table 1.

Recoveries were satisfactory for an Australian blended brandy sample spiked with KCN at various levels and analyzed by the same procedure (Table 1). A blank determination on the brandy showed no peak corresponding to CNBr. A sample of brandy which had previously been analyzed for ethanol and the other major congeners by method **9.075** (3) was also subjected to the same isolation procedure. The contents of the NaOH absorber trap were then analyzed for the presence of congeners but only ethanol and methanol were detected in significant quantities.

However, we obtained low recoveries (31–52%) for a wine sample known to contain SO_2, spiked with 10 μg CN^-/200 ml (0.05 ppm) as KCN, and analyzed for HCN by the same method used for the brandy samples. In this case, the bromide water was completely decolorized when added to the contents of the NaOH trap. This did not occur for brandy samples. Adding excess bromine water did not improve these recoveries. The problem appeared to be due to SO_2 in the sample. Bates (1) previously reported that SO_2 interfered in the development of the color complex formed in the qualitative method for the detection of cyanide. This interference was removed by introducing a lead dioxide column between the condenser and the test paper as specified in **9.102(f)** and **11.053** (3).

A number of experiments were then performed

in a similar manner on wine samples known to contain SO_2 and spiked with KCN except that liberated HCN was first passed through a PbO_2 column prior to absorption in the 0.1*N* NaOH trap. Reasonably precise results and better recoveries were obtained. In 4 tests of 10 μg CN^-/200 ml wine (0.05 ppm), the mean recovery was 72%, range 6.8–7.5 μg, standard deviation 0.3 μg, and relative standard deviation 4.6%. Distilling for longer periods did not significantly increase recoveries. We suspected some of the HCN may have been trapped in the PbO_2 column. In further experiments, spiked wine samples were first distilled 2 hr through the PbO_2 column in the above manner, the original NaOH trap was then replaced with one containing fresh NaOH solution, and the contents of the PbO_2 column were added to the original acidified wine in the distillation flask. The distillation was restarted and proceeded for 2 hr. Additional HCN (10–20%) was recovered, so some HCN had been trapped in the PbO_2 column, but the results of these additional recoveries were somewhat erratic. This may be due to a number of factors including the flow rate of the nitrogen and the particle size of the PbO_2.

A method that seemed more promising for removing SO_2 from the system was then investigated and is reported in detail below under *Isolation of Cyanide from Samples Containing Sulfur Dioxide*. The cyanide in the sample is first fixed by reacting it with mercuric chloride in the distillation flask. The mercuric cyanide formed is very stable and is practically undissocompletely removed by the technique of heating at 70°C and flushing with gas after which the mercuric cyanide is dissociated by adding KI (10) and the liberated HCN is removed in the manner described above. Our investigations of this procedure indicated that the acidified sample in which cyanide had been converted to mercuric cyanide should not be boiled during the removal of SO_2, because low recoveries were obtained.

METHOD

Apparatus

(Assembled as shown in Fig. 1. These items are also specified in ref. 3, 9.101(a), (c), and (d).)

(a) *Distilling flask.*—500 ml, round-bottom, 3-neck with ground socket joints, angle-type (1963 Fisher No. 10-165, or equivalent).

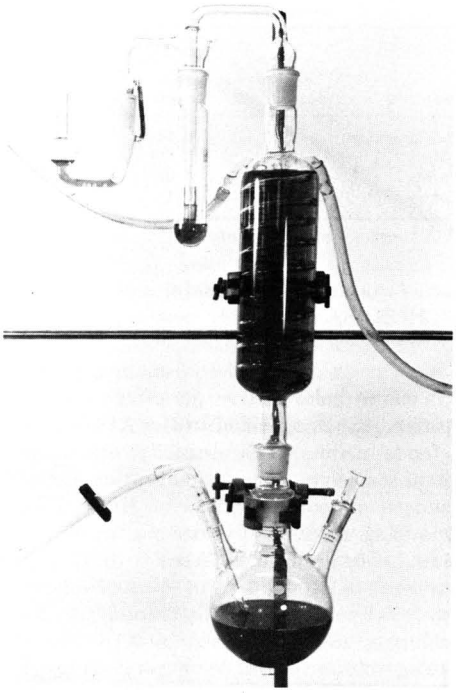


FIG. 1—Distillation and trap apparatus for recovery of cyanide from alcoholic beverages.

(b) *Nitrogen inlet tube.*—With cone to fit side neck of distilling flask and long enough to almost reach bottom of flask.

(c) *Condenser.*—Graham coil-type, with inner and outer joints, cone to fit center neck of distilling flask (1963 Fisher No. 7-728, 400 mm jacket length, or equivalent), and cooled with flow of cool water.

(d) *Stopper.*—With cone to fit side neck of distilling flask.

(e) *Gas absorber/washer.*—It consists of 2 parts: bubbling tube immersed in absorbing solution in 50 ml test tube which is connected to U-tube also containing absorbing solution plus 2–3 glass beads to break up bubbles, as shown in Fig. 1. (*See also* the Fisher-Milligan gas washer specified in ref. 9 and gas absorbers specified in ref. 3, 20.102.)

(f) *Gas chromatograph.* — Varian Aerograph Model 1200 gas chromatograph equipped with ^3H source concentric design electron capture detector and coupled to a 1 mv chart recorder. Applied voltage 90 v dc, sensitivity 1×10^{-10} amp/mv, and attenuation ca ×128. A 6′ × ⅛″ od stainless steel

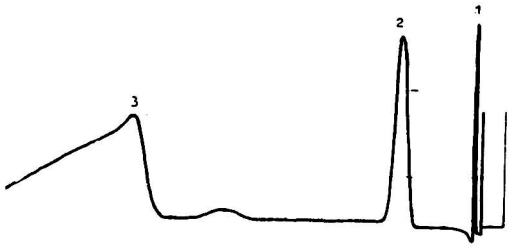


FIG. 2—Gas chromatogram of CNBr recovered from brandy spiked with KCN (2 μg CN^-/200 ml, 0.01 ppm) by gas flushing HCN into NaOH absorber solution, brominating, and extracting CNBr with diisopropyl ether; 5 μl injected. 1, water; 2, CNBr; 3, $(C_3H_7)_2O$.

column packed with 50–80 mesh Porapak Q and glass-lined injector port was used. Operating parameters: nitrogen carrier gas ca 50 ml/min, column 125°C, detector 180°C, injector 170°C, chart speed 5 mm/min.

(g) *Constant temperature water bath.*—Suitable for immersing 500 ml flask and capable of maintaining 70±2°C.

Reagents

(All chemicals were analytical reagent grade.)

(a) *Sodium hydroxide.*—0.1*N* solution.

(b) *Mercuric chloride.*—2% solution.

(c) *Stock cyanide solution.*—Dissolve 1.251 g KCN in 500 ml water (1000 μg CN^-/ml). Standardize against $AgNO_3$ solution, using *p*-dimethylaminobenzalrhodamine indicator as described in *Standard Methods for the Examination of Water and Wastewater* (9).

(d) *Standard cyanide solution.*—Dilute 5 ml stock solution to 500 ml with water, mix, and dilute 10 ml to 100 ml. Prepare final dilution fresh daily (1 ml = 1.0 μg CN^-).

(e) *Sulfuric acid.*—1+9.

(f) *Orthophosphoric acid.*—20% solution.

(g) *Phenol.*—5% solution.

(h) *Diisopropyl ether.—Caution:* This may form explosive peroxides on standing and should periodically be passed through an activated alumina column.

Isolation of Cyanide from Samples Free of Sulfur Dioxide

Control recovery test using standard KCN solution.—Place 200 ml alcohol-water (1+1) in distilling flask which is immersed in 70°C constant temperature water bath. Assemble apparatus as shown in Fig. 1. Connect absorber trap, with ca 8 ml and 2 ml 0.1*N* NaOH in large test tube and U-tube, respectively, to top of condenser. Add aliquot of standard KCN solution (1 μg/ml) to flask, plus 10 ml H_2SO_4 (1+9) through side neck. Replace stopper. Pass stream of nitrogen gas through liquid in flask and up through condenser. HCN carried with it is absorbed in NaOH solution in trap. Continue 2 hr.

Adjust bubbling rate to give discrete bubbles so NaOH does not splash out of absorber. Add contents of U-tube to solution in test tube, rinsing the former with 2–3 ml water. When cyanide content is less than 20 μg CN^-, total volume in absorber should not exceed 25 ml. For higher levels of cyanide, solution can be diluted to 50 ml in volumetric flask and an aliquot can be analyzed. Proceed with *Gas Chromatography* below. Analyze blank concurrently.

Samples.—Use same procedure for suitable aliquot of sample known to be free of SO_2.

Isolation of Cyanide from Samples Containing Sulfur Dioxide

Control recovery test using standard KCN solution.—Assemble apparatus as shown in Fig. 1 except do not place absorber trap on top of condenser until indicated below. Place 200 ml alcohol-water (1+1) in distilling flask which is immersed in *room temperature* water bath. Add aliquot of standard potassium cyanide solution (1 μg CN^-/ml) to flask plus 2 ml 2% mercuric chloride solution and bubble nitrogen gas through solution 15 min.

Increase temperature of water bath to 70°C as quickly as possible. Add 10 ml H_2SO_4 (1+9) and pass nitrogen through solution 1 hr. Turn off nitrogen, connect NaOH absorber trap with condenser, add ca 1 g KI to contents of distillation flask through side neck, replace stopper in side arm of flask, and continue to pass nitrogen through system additional 2 hr.

Add contents of U-tube to test tube, rinsing former with water as described above. Proceed with *Gas Chromatography*. Analyze blank concurrently.

Samples.—Use same procedure on a suitable aliquot of wine or other sample known to contain SO_2.

Gas Chromatography

Samples.—Add 2 ml 20% orthophosphoric acid and 12 drops saturated bromine water (ca 0.5 ml) to test tube containing alkaline HCN solution, stopper, mix, and let stand 15 min. Add 0.2 ml 5% phenol solution, mix, pipet in 5 ml diisopropyl ether, and shake 2 min. Let layers separate, withdraw aliquot (usually 5 μl) of ether phase into syringe, and inject onto column. Retention time of CNBr is ca 2 min, 45 sec under these conditions (Fig. 2). Measure area under CNBr peak and

Table 2. Recoveries of CN^- added as KCN to 200 ml wine, using the method of isolation of HCN from samples containing SO_2, and gas chromatography

Added μg	ppm	No. of tests	Recovery				
			Mean,[a] μg	Range, μg	Std dev.	Rel. std dev., %	Av., %
2.0	0.01	5	1.3	1.2– 1.4	0.01	0.8	65
10.0	0.05	5	9.0	8.3– 9.6	0.5	5.2	90
20.0	0.1	5	18.1	15.6–19.8	1.9	10.2	91

[a] Outliers were not excluded.

compare with area produced from known amount of standard KCN solution, obtained as follows:

Standards.—Take aliquot of standard KCN solution that will produce CNBr peak of approximately same area as that for sample. Transfer to 7 test tube, add 10 ml 0.1*N* NaOH, 2 ml 20% phosphoric acid, and 12 drops saturated bromine solution, mix, and let stand 15 min. Add 0.2 ml 5% phenol solution, mix, pipet in 5 ml diisopropyl ether, shake 2 min, and let layers separate. Inject aliquot (usually 5 μl) of ether phase onto column and measure area of CNBr peak obtained.

Calculations.—

$$CN^-,\ ppm = [(A - A_b)/A'] \times (V'/V_x) \times (W'/V)$$

where A = peak area of sample; A_b = peak area of blank; A' = peak area of standard; V_x = injected volume of sample extract in μl; V' = injected volume of standard extract in μl; W' = weight of CN^- in standard in μg; and V = volume of original sample. It is recommended that V_x and V' be kept equal. The volume of injected blank extract must be equal to V_x.

Results and Discussion

The quantitative isolation of HCN from alcoholic beverages in a form suitable for the determinative step presents more problems than it does for water samples, particularly if SO_2 is present in the product.

The recovery results obtained from replicate analyses of distilled liquors, free of SO_2, spiked with CN^- at 2–20 μg, are given in Table 1. They range from 80 to 97% and are equal to or better than the minimum recovery level of 80% that is generally acceptable for residue analyses (11, 12). The precision indicated by the relative standard deviations shown is considered to be quite acceptable.

The recovery results obtained from replicate analyses of wine samples known to contain significant amounts of SO_2 which had to be removed prior to the determinative step are given in Table 2. A similar degree of accuracy and precision was obtained at the 10 and 20 μg level, but these were not quite so good at the 2 μg level. Blank determinations were performed for all wines and the blank values were subtracted from the total recovered.

The limit of detection of the GC determination, defined as that amount which will give a peak height greater than twice the size of baseline fluctuations (noise, etc.), was 0.2 μg HCN. This was determined by serially diluting aliquots of standard KCN solution, converting the cyanide to CNBr in each diluted solution by the procedure given for the preparation of standards, extracting into diisopropyl ether, and injecting 5 μl.

The identity of the CNBr peaks obtained from standard solutions of KCN was confirmed at the outset of these investigations by comparing with the retention time of peaks obtained from a solution of solid CNBr (E. Merck) dissolved in petroleum ether, under the same operating parameters. This solid reagent rapidly decomposes in contact with air and has a limited shelf life.

All diisopropyl ether extracts of CNBr obtained from spiked samples and standards were injected in triplicate for greater precision because this was a special study. The mean of the areas was taken for the calculations. However, the reproducibility of the gas chromatographic system was very good and this replication would not be necessary for routine determinations.

In general, distilled products such as rectified spirits, whisky, brandy, vodka, rum, and gin are free from SO_2, whereas table wines invariably contain significant amounts of SO_2. If there is any doubt, samples should be subjected to the qualitative test for sulfurous acid, 20.100 (3).

The GC determinative step was more rapid and reliable and required fewer manipulations

than the colorimetric procedure. We propose to use the rapid qualitative screening test given in *Official Methods of Analysis* (3) to initially detect the presence of cyanide in samples and, if positive results are obtained, determine the amount of HCN quantitatively by the above method. The same basic apparatus can be used for both tests, except that the ground-glass flange assembly containing the test papers used for the qualitative test will be replaced by the absorber assembly containing 0.1*N* NaOH.

It is recommended that the method be studied further.

References

(1) Bates, B. (1970) *JAOAC* **53**, 775–779
(2) Bates, B. (1973) *JAOAC* **56**, 840–842
(3) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, secs. **9.101–9.103**; **11.053**
(4) Sunshine, I. (Ed.) (1969) *Handbook of Analytical Toxicology,* Chemical Rubber Company Publishers, Cleveland, OH, pp. 1015–1018
(5) Kruse, J., & Thibault, L. (1973) *Anal. Chem.* **45**, 2260–2261
(6) Nota, G., & Palombari, R. (1973) *J. Chromatogr.* **84**, 37–41
(7) Valentour, J., Aggariwal, V., & Sunshine, I. (1974) *Anal. Chem.* **46**, 924–925
(8) Epstein, J. (1947) *Ind. Eng. Chem. Anal. Ed.* **19**, 272–273
(9) Taras, M., *et al.* (Eds.) (1971) *Standard Methods for the Examination of Water and Wastewater,* 13th Ed., American Public Health Association Publishers, Washington, DC, pp. 400–403
(10) Feigl, F. (1958) *Spot Tests in Inorganic Analysis,* 5th Ed., Elsevier Publishing Co., Amsterdam, The Netherlands, pp. 280–281
(11) Zweig, G., & Sherma, J. (1972) *Handbook of Chromatography,* Vol. II, Chemical Rubber Company Publishers, Cleveland, OH, p. 243
(12) *Pesticide Analytical Manual* (1964) Vol. 1, Food and Drug Administration, Washington, DC, sec. **201.1**

The recommendation of the Associate Referee, B. L. Bates, was approved by the General Referee and by Subcommittee D and was accepted by the Association. *See* (1976) *JAOAC* **59**, 388.

CEREAL FOODS

Loss of Chloride in the Official Method for the Determination of Sodium Chloride in Cereal Foods

WALLACE S. BRAMMELL
Division of Color Technology, Food and Drug Administration, Washington, DC 20204

The official final action method for sodium chloride in cereal foods, 14.129, was found to give erroneously low results because of loss of chloride during ashing. Comparison of the data with values obtained by the official first action potentiometric method, 32.A01–32.A06, which does not require ashing, showed that large and variable losses of chloride occurred. Official ashing methods for other foods specify addition of sodium carbonate to prevent conversion of chloride to volatile forms, but this was not specified in 14.129. In the present study it was found that sodium carbonate did not completely prevent loss of chloride. The official first action potentiometric method, 32.A01–32.A06, has been adopted as official first action for the determination of chloride in cereal foods to replace 14.129, which was repealed, official first action. A cross-reference to 32.A01–32.A06 has been added to 14.096.

In order to remove organic matter that could interfere in the determination of chloride in foods, official AOAC methods (1) have usually specified either wet ashing with nitric acid or dry ashing in a muffle furnace. Dry ashing is employed in official methods for the determination of chloride in plants, **3.067–3.070**; beer, **10.067**; wines, **11.026**; bread, **14.096**; macaroni, egg noodles, and similar products, **14.129**; roasted coffee, **15.019**; butter, **16.190**; eggs and egg products, **17.025**; fruits and fruit products, **22.051**; meat extracts and similar products, **24.061**; nuts and nut products, **27.012**; and canned vegetable products, **32.017**.

To prevent loss of chloride by conversion to a volatile form during ashing, these methods generally specify addition of a fixative. In 8 of the 12 methods listed above, sodium carbonate is added for this purpose, usually 20 ml 5% solution as given in **3.067**. In method **27.012**, 10 ml 10% $Ca(OAc)_2$ solution is used. In the other 3 methods (**14.096**, **14.129**, and **16.190**), the use of a fixative is not specified.

The addition of a fixative to prevent loss of chloride during ashing may not be required under certain conditions, i.e., (*1*) low carbohydrate content of sample, (*2*) low ratio of carbohydrate to chloride, (*3*) low ashing temperature, (*4*) short ashing time, and (*5*) formation of an alkaline ash. As the carbohydrate content of cereal foods is often very high and no provision is made for adding a fixative such as sodium carbonate, it seemed likely that loss of chloride would occur when samples are ashed by the official final action method, **14.129**. Therefore, one purpose of this investigation was to determine whether such loss does take place and, if so, the magnitude of the loss. Another purpose was to determine if adding sodium carbonate prevented this loss. Osborn (2) demonstrated the need for a fixative to prevent loss of chloride in ashing fruit products, even though as he states ". . . fruit ash normally contains approximately 75 per cent of potassium carbonate, which should, and no doubt does, act to reduce loss of chlorine during ashing."

With regard to the problems involved in ashing samples for chloride determination, Browne (3) in his AOAC President's Address called attention to the inadequacy of AOAC methods for chloride in plant and animal products because of the loss that can occur during incineration, even with the addition of sodium carbonate. In 1928, Tilden (4) demonstrated that large losses of chloride occurred when sucrose and various foods, including cheese, oysters, and fruits, were ashed without a fixative. She concluded that sodium carbonate in a ratio of at least 5 parts to 1 part of chloride should be added before ashing. As a result of this work, the addition of 20 ml 5% Na_2CO_3 to samples before ashing was specified in **3.067**, the basic dry ashing method for chloride.

In 1934, Stone (5) conducted a collaborative study of the official method for the determina-

tion of salt in cheese after it was brought to his attention that it gave erroneously low results. In this study the collaborators ashed samples according to the official method which specified no fixative, and ashed samples with calcium acetate or sodium carbonate added as fixatives; they also used wet ashing with nitric acid and potassium permanganate solution. The loss of chloride from 5 samples of natural cheese analyzed by the official method averaged about 24%. However, the loss from 3 samples of processed cheese averaged only 2%. The author attributed this low loss to the presence of an emulsifier which acted as a base. The sodium chloride values obtained for natural cheese with calcium acetate were almost identical with those obtained with sodium carbonate and wet ashing. However, the method was deleted the following year and replaced by the wet ashing method.

Pickett (6) investigated the effect of ashing temperature on losses of chloride from various plant and animal products. When samples weighing approximately 5 g were ashed with 20 ml 5% Na_2CO_3 added, he found that there was little, if any, chloride lost at 500 and 600°C. However, in some samples there was a loss of chloride at 700°C; at 800°C the loss was considerable for most of the materials tested. He found that even at 500°C there was a large loss of chloride from some samples if sodium carbonate was not added.

In a collaborative study of a potentiometric titration method for determining sodium chloride in foods, Brammell (7) obtained data on a wide variety of foods. The results indicated that when 5 g samples were ashed with 1 g Na_2CO_3 (20 ml 5% Na_2CO_3 solution) as specified in **3.067**, there was little or no loss of chloride.

A review of the history of the 50-year-old method for determining sodium chloride in cereal foods, **14.129**, is required to help explain why the recommendation for its deletion came so late. This method was adopted as a tentative (official first action) method in 1925 and was made official (official final action) in 1926 for alimentary pastes (later changed to macaroni, egg noodles, and similar products) without a collaborative study. In 1928 it was adopted by cross-reference as a tentative method for chloride in baked products (later changed to bread, method **14.096**), again without collaborative study. In 1929 (8), the General Referee recommended that the method be studied collaboratively for the purpose of adopting it as official final action, but the study was never done.

In 1934 (9), the Associate Referee proposed a method for determining the salt-free ash of macaroni products and baked products. In this method the weight of added sodium chloride, determined by **14.129**, was subtracted from the weight of the ash. It soon became apparent that this method did not give accurate values for salt-free ash. The Associate Referee, in his 1939 (10) and 1940 (11) reports, attributed this to loss of chloride during ashing, but he presented no data showing the magnitude of the loss.

In his 1940 report (12), the General Referee recommended that the tentative method for bread (now **14.096**) be adopted as official final action, although the collaborative study proposed in 1929 had never been conducted. In his 1942 report (13), the General Referee stated "Section **91**, page 235, entitled 'Chloride in Ash as Sodium Chloride, Official' [now **14.129**] cannot be satisfactorily used for the correction of added sodium chloride in the estimation of the original ash of macaroni products owing to the decomposition of the added sodium chloride and subsequent loss of chlorine during ashing." Apparently the determination was performed because of the convenient availability of the original ash. The inaccuracy was acknowledged in the parenthetical statement at the end of method **14.129** in each edition of *Official Methods of Analysis* beginning with the fourth edition in 1935. It originally read "(This NaCl value deducted from the total ash does not give NaCl-free ash)." In the seventh edition (1950) this was changed to read "(This NaCl value deducted from total ash gives only approximate NaCl-free ash)." A more useful and pertinent statement would have been "(This method gives only approximate values for NaCl)."

Experimental

The samples were prepared for analysis, without any preliminary grinding, by preparing water suspensions in a Waring Blendor and preserving them with formaldehyde solution as described in the official first action potentiometric method **32.A01–32.A06** (7, 14), using a 50 g sample and 450 g water. The NaCl content of the samples was then determined by titrating 50 g suspension with $AgNO_3$ solution to the potentiometric end point, **36.A06**.

To determine the effect of ashing on the NaCl content of the sample, 50 g (equivalent to 5 g original sample) of the same suspensions previously analyzed by potentiometric titration were weighed in tared 250 ml Pyrex beakers. Some samples were spiked with NaCl, using the standard solution specified in **32.A03(c)**. This solution contains 5 g NaCl/L, so 1 ml was added for each 0.1% additional NaCl desired in the sample. The sample suspensions were analyzed as in **14.129** with no fixative added, with 1 g Na_2CO_3 (20 ml 5% solution as specified in **3.067**) added, and with 2 g Na_2CO_3 (40 ml 5% solution) added. These suspensions were mixed and heated to dryness overnight in a 130°C oven.

After drying, the beakers were placed in a cool muffle furnace and heated for 2, 4, or 6 hr at 550°C or for 2 hr at 650°C. After the beakers were cooled to room temperature, 5 ml water was added to wet the residue in beakers with no Na_2CO_3 and with 1 g Na_2CO_3. Five ml HNO_3 (1+4) was added to the 2 g Na_2CO_3 beakers to neutralize the additional 1 g Na_2CO_3. Most of the beakers contained a considerable amount of carbon which was pulverized with a stirring rod. Then 100 ml HNO_3 (1+19) was added to each beaker, and the chloride was titrated with $AgNO_3$ solution to the potentiometric end point as described in ref. 7, *Preliminary Study*, sec. (b).

The oatmeal sample was also analyzed without first preparing a suspension. Three 5 g portions of the dry cereal were weighed into 250 ml beakers and spiked with NaCl solution; 0, 20, and 40 ml 5% Na_2CO_3 were added. The samples were mixed, dried overnight in a 130°C oven, ignited over a Meker burner, and then ashed in a muffle furnace for 2 hr at 550°C.

The loss of chloride during ashing was calculated as follows:

$$\text{Chloride loss, \%} = [(\text{\% NaCl in unashed sample} - \text{\% NaCl in ashed sample}) / \text{\% NaCl in unashed sample}] \times 100$$

Results and Discussion

The data in Table 1 show that the official final action method for the determination of sodium chloride in cereal foods, **14.129**, gave low results for bread and macaroni products, cereals, and snack foods because of loss of chloride during ashing. This was demonstrated by comparing the apparent sodium chloride values obtained by **14.129** with those found by using the official first action potentiometric method, **32.A01–32.A06**, which does not require ashing. The loss of chloride during ashing of bread samples ranged from 18.2 (rye bread) to 34.5% (whole wheat bread). For macaroni products, the loss ranged from 66.2% for macaroni containing 0.3% added salt to 94.3% for the same macaroni without added salt. For cereals, the loss of chloride was 28.0% for oat cereal and 92.6% for oatmeal with 0.3% NaCl added when the samples were ashed 2 hr at 550°C; increased losses occurred with a 6-hr ashing at 550°C. The losses from the 2 snack foods tested were somewhat lower; crackers lost 14.8 and 16.8% when ashed at 550°C for 2 and 6 hr, respectively. Pretzels were ashed for 6 hr at 550°C and lost 3.9% of the chloride. The sodium carbonate added during the manufacture of pretzels probably accounts for the small loss of chloride found. Addition of sodium carbonate before ashing eliminated loss of chloride from bread samples and pretzels; loss in the other samples studied was lowered but, in many cases, the loss was still high (*see* Table 1). Therefore, even if **14.129** had called for ashing according to **3.067** rather than **14.006**, it would not have yielded results that were accurate enough for an official method.

The samples ashed without added sodium carbonate still contained some carbon, even after 6 hr. If they had been ashed longer to obtain a light gray ash or to constant weight as directed in **14.129**, the data in Table 1 suggest that even higher losses of chloride would have occurred.

Some interesting data were obtained when 5 g samples of oatmeal were ashed without first preparing a suspension in a Waring Blendor. These samples were spiked with 0.1, 0.3, and 0.5% NaCl and ashed 2 hr at 550°C. Comparison of the results obtained for the sample spiked with 0.3% NaCl with the comparable one prepared as a suspension shows that the loss from the coarse, unblended sample was somewhat lower (77.6 vs. 92.6%) when the samples were ashed without sodium carbonate for 2 hr at 550°C. But the loss from the coarse sample ashed with 1 g Na_2CO_3 for 2 hr at 550°C was nearly as great as for the blended sample after a 6 hr ashing at 550°C, and the loss from the coarse sample was somewhat higher with 2 g Na_2CO_3 added. Therefore, it appears that sample particle size may be another factor affecting loss of chloride during ashing, although the preashing with a burner may have contributed to the loss. The data also show that, with the un-

Table 1. Loss of chloride in the determination of sodium chloride in cereal food samples

Sample	% NaCl[a]					Loss of chloride, %		
		Ashed						
	32.A06, no ashing	14.129, no Na_2CO_3	1 g Na_2CO_3	2 g Na_2CO_3	Time,hr	14.129, no Na_2CO_3	1 g Na_2CO_3	2 g Na_2CO_3
Bread								
White bread	1.71	1.30	1.71	1.72	6	24.0	0.0	0.0
Rye bread	1.37	1.12	1.38	1.39	2	18.2	0.0	0.0
Whole wheat bread	1.21	0.792	1.21	1.21	2	34.5	0.0	0.0
Macaroni Products								
Egg noodles	0.130	0.036	0.127	0.132	2	72.3	2.3	0.0
Egg noodles + 0.5% NaCl	0.640	0.207	0.635	0.640	2	67.7	0.8	0.0
Macaroni	0.088	0.005	0.111	0.101	2	94.3	0.0	0.0
Macaroni + 0.3% NaCl	0.388	0.131	0.361	0.379	2	66.2	7.0	2.3
Cereals								
Oat cereal, ready-to-eat	3.36	2.42	2.84	2.90	2	28.0	15.0	13.7
	3.36	2.34	2.86	2.87	4	30.4	14.9	14.6
	3.36	2.29	2.88	2.86	6	31.8	14.3	14.9
	3.36	2.22	2.89	2.87	2[b]	33.9	14.0	14.6
Oatmeal + 0.3% NaCl	0.380	0.028	0.380	0.383	2	92.6	0.0	0.0
	0.380	0.010	0.368	0.371	4	97.4	3.2	2.4
	0.380	0.008	0.350	0.371	6	97.9	7.9	2.4
	0.380	0.001	0.376	0.369	2[b]	99.7	1.1	2.9
Oatmeal + 0.1% NaCl	0.180	0.035	0.161	0.174	2	80.6	10.6	3.3
Oatmeal + 0.3% NaCl	0.380	0.085	0.351	0.369	2	77.6	7.6	2.9
Oatmeal + 0.5% NaCl	0.580	0.151	0.540	0.572	2	74.0	6.9	1.4
Snack Foods								
Crackers	1.55	1.32	1.54	1.54	2	14.8	0.6	0.6
	1.55	1.31	1.54	1.54	4	15.5	0.6	0.6
	1.55	1.29	1.54	1.54	6	16.8	0.6	0.6
	1.55	1.21	1.54	1.53	2[b]	21.9	0.6	1.3
Pretzels	4.55	4.37	4.56	4.57	6	3.9	0.0	0.0

[a] Single determinations on samples ashed at 550°C.
[b] Ashed at 650°C.

blended oatmeal spiked with sodium chloride, the per cent loss of chloride during ashing was inversely related to the initial chloride content.

Conclusions and Recommendations

The official final action method for the determination of sodium chloride in cereal foods, **14.129**, was found to give low results because of failure to specify addition of a fixative to prevent loss of chloride during ashing. However, it was shown that erroneously low results are also obtained for some cereal foods using the amount, or even twice the amount, of sodium carbonate recommended in similar official methods for other foods.

It is recommended (*1*) that the official first action potentiometric method, **32.A01–32.A06**,[1] be adopted as official first action for the determination of chloride in cereal foods to replace **14.129**, which should be repealed, official first action; and (*2*) that a cross-reference to **32.A01–32.A06** be added to **14.096** to replace the present cross-reference to **14.129**.

REFERENCES

(1) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC
(2) Osborn, R. A. (1943) *JAOAC* **26**, 437–440
(3) Browne, C. A. (1926) *JAOAC* **9**, 15–23
(4) Tilden, D. H. (1928) *JAOAC* **11**, 209–218

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee D and were adopted by the Association; *see* (1976) *JAOAC* **59**, 388.

[1] This method was adopted as an official final action method for processed vegetable products at the 1975 AOAC meeting; *see* "Changes in Methods" (1976) *JAOAC* **59**, 473.

(5) Stone, C. B. (1934) *JAOAC* **17**, 347–350
(6) Pickett, T. A. (1938) *JAOAC* **21**, 107–108
(7) Brammell, W. S. (1974) *JAOAC* **57**, 1209–1216
(8) Le Clerc, J. A. (1930) *JAOAC* **13**, 433–438
(9) Bailey, L. H. (1935) *JAOAC* **18**, 562–563
(10) Bailey, L. H. (1939) *JAOAC* **22**, 522–523
(11) Bailey, L. H. (1940) *JAOAC* **23**, 480–482
(12) Munsey, V. E. (1940) *JAOAC* **23**, 477–478
(13) Munsey, V. E. (1942) *JAOAC* **25**, 618–621
(14) "Changes in Methods" (1975) *JAOAC* **58**, 399–400

Received September 11, 1975.

This report of the Associate Referee was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

DRUGS

Differential Pulse Polarography of Cacodylate Injections

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

A method was developed for the analysis of cacodylate injections, using differential pulse polarography for measuring As(III). The cacodylate molecule is decomposed to As(V) by heating with $Mg(NO_3)_2.6H_2O$; As(V) is then chemically reduced to As(III) and polarographed in 1*M* HCl supporting electrolyte. The method of standard addition is used for quantitation. Recoveries based on the analysis of synthetic cacodylic acid solutions containing approximately 9 mg arsenic/ml ranged from 99.1 to 101.7%.

The established methods for the analysis of cacodylates are based on iodometric (1) or bromate (2) titrations after digestion of the sample with sulfuric acid. In order to take advantage of modern instrumentation, a procedure (3) was developed in which the cacodylate moiety was measured by nuclear magnetic resonance spectroscopy and total arsenic was determined by atomic absorption spectrophotometry. The determination of arsenic by atomic absorption spectrophotometry, however, was not entirely satisfactory because of well known interferences which result from the position of the resonance lines of arsenic in the short wavelength region of the ultraviolet spectrum. In this region, the atmosphere as well as the combustion products of an air-acetylene flame absorb strongly.

Myers and Osteryoung (4) reported that arsenic can be determined readily by differential pulse polarography. In this report a simple, accurate, and precise method is described for the analysis of cacodylate injections by measuring arsenic as As(III) by differential pulse polarography. The standard addition technique is useful for pharmaceutical dosage forms where unknown components are present; with this technique the components will exert the same effect on the sample system as on the standard.

METHOD

Apparatus and Reagents

All reagents are reagent grade, unless otherwise indicated.

(a) *Polarograph.*—PAR Model 174 (Princeton Applied Research Corp., Princeton, NJ 08540), or equivalent. Typical instrument settings: scan rate 2 mv/sec, scan direction —, range 1.5 v, initial potential —0.15 v, modulation amplitude 50 mv, operation mode differential pulse, current range, 0.05 ma, or as needed, output offset as required, display direction +, drop time 1 sec, low pass filter off, selector off, pushbutton initial, recorder: *x*-axis, 0.1 v/in., *y*-axis, 1 v/in.

(b) *Cells.*—Standard cell bottom (PAR No. 9301), saturated calomel reference electrode (SCE), carbon rod counter electrode, and dropping mercury indicating electrode.

(c) *Pipet.*—100 μl Eppendorf, or equivalent (Princeton Applied Research Corp.).

(d) *Supporting electrolyte.*—1*M* HCl. Add 82 ml concentrated HCl to ca 500 ml water in 1 L volumetric flask, dilute to volume with water, and mix.

(e) *Arsenic standard solution.*—2.000 mg/ml As(III). Dissolve 2.640 g As_2O_3, dried 1 hr at 105°C, in ca 25 ml 1*N* NaOH in 1 L volumetric flask, acidify to litmus paper with 1*N* HCl, and dilute to volume with water.

Digestion of Sample (5)

Transfer to 100 ml borosilicate beaker accurately measured volume of injection, diluted if necessary, containing ca 29 mg sodium cacodylate (ca 10 mg As), and add 1.0 g $Mg(NO_3)_2.6H_2O$ and 1 ml concentrated HNO_3. Heat on hot plate at low heat until water is evaporated and then use high heat to obtain solid residue. Complete digestion by placing beaker in 400°C muffle furnace until no brown fumes are evolved (ca 30 min). Remove from furnace and let cool to room temperature.

Chemical Reduction of As(V) to As(III) (6)

Add 20 ml 5*M* HCl to residue in beaker and swirl to dissolve, warming on steam bath, if necessary; then add 2 ml 40% HBr and 0.3 g hydrazine sulfate. Cover beaker with watch glass and place on steam bath 30 min. Cool to room temperature, transfer with water to 100 ml volumetric flask, dilute to volume, and mix.

Received September 5, 1975.

Determination

Add 20 ml supporting electrolyte to polarographic cell and pipet 2 ml sample solution into cell. Bubble nitrogen through solution 5 min; then direct gas to sweep over solution. Switch selector to "Cell" and allow enough time for recorder pen to come to rest. Depress "Scan" pushbutton and record polarogram from −0.15 to −0.9 v. Add 100 µl standard solution to cell and bubble nitrogen through solution 1 min; then direct gas to sweep over solution and record polarogram as before, using same instrumental settings. Repeat this procedure on 2 additional successive 100 µl aliquots of standard solution. Plot standard addition curve as follows: mg standard added (0, 0.2, 0.4, and 0.6) on x-axis and first As peak height which appears at ca −0.36 v vs. SCE on y-axis. Extrapolate linear plot to x-axis to obtain mg As in sample. As is shown in Fig. 1, standards need not be polarographed beyond ca −0.60 v, since analytical peak is at ca −0.36 v vs. SCE.

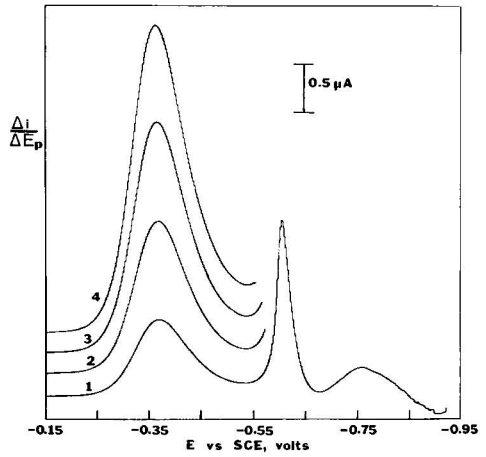


FIG. 1—Differential pulse polarograms of As(III) in 1*M* HCl. 1, 1*M* HCl (20 ml) + solution (2.00 ml) derived from cacodylic acid and exhibiting features of arsenic reductions; 2, Solution 1 + 0.200 mg As(III) (0.100 ml); 3, Solution 2 + 0.200 mg As(III) (0.100 ml); 4, Solution 3 + 0.200 mg As(III) (0.100 ml).

Results and Recommendation

The method was tested by using both differential pulse polarography and iodometric titration to analyze synthetic solutions prepared from cacodylic acid; *see* Table 1. The polarographic results indicate that this method is both accurate and precise. In addition, comparison of the polarographic results with those from iodometric titration shows excellent agreement. A single commercial sodium cacodylate injection labeled as containing 320 mg/5 ml was also analyzed in duplicate by both procedures and the results again were in excellent agreement: 319.6 and 317.8 mg/5 ml (polarography) and 319.6 and 316.6 mg/5 ml (titration). The differential pulse polarographic procedure is simpler and faster, thus making it more attractive.

The differential pulse polarographic method offers several other advantages for the analysis of cacodylates. First, As(III) in 1*M* HCl produces a characteristic polarogram consisting of 2 peaks which correspond to the reduction of As(III) to As^0 (−0.36 v) and As^0 to AsH_3 (−0.75 v) as well as a maximum (−0.60 v). The characteristic polarogram offers a convenient identity test for arsenic (Fig. 1). Second, oxidation states of arsenic or its presence in organic compounds can be distinguished. For example, if one wishes to check for the presence of As(III), the solution can be made 1*M* in HCl and polarographed. Following this, As(V) can be determined after chemical reduction to As(III). Under the conditions employed, As(V) is not polarographically active at the dropping mercury electrode, which is also true for arsenic organically bound as cacodylate. Thus inorganically bonded arsenic can be differentiated.

Linearity is an important consideration. In this study the observed current was linearly related to the concentration even at the highest value, 40 µg/ml. The response is known to be linear up to about 60 µg/ml (4), above which nonlinear behavior is observed.

Since some cacodylate dosage forms occur as the ferric salt, the interference of iron must be anticipated. Both the iodometric (2) and bromate (4) titrations cannot yield stoichiometric results in the presence of iron. Therefore, arsenic must be separated first by distillation as $AsCl_3$. However, in the proposed method, iron would be present as Fe(II) and would not interfere because Fe(II) is reduced at a more negative potential.

The conditions under which electrochemical analysis is conducted affect the quality of analytical results. In this case the rate of scan was

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee B and was accepted by the Association. *See* (1976) *JAOAC* **59**, 382.

Table 1. Analysis of cacodylic acid solution

	Differential pulse polarography		Iodometric titration (1)	
Detn	Found, mg/ml	Rec., %	Found, mg/ml	Rec., %
1	8.98	99.6	8.97	99.6
2	9.03	100.2	9.02	100.1
3	8.93	99.1		
4	8.93	99.1		
5	9.16	101.7		
6	8.93	99.1		
Av.	9.00	99.9	9.00	99.8
Range	8.93–9.16	99.1–101.7	8.97–9.02	99.6–100.1
Mean and std dev.	9.00±0.091	99.9±1.02	9.00±0.035	99.7±0.35
Rel. std dev., %	1.01	1.03	0.39	0.36

found to be important in that a 2 mv/sec rate yielded more precise results than a 5 mv/sec rate.

The polarographic method described here has a number of other advantages. The curve characteristics identify the form of arsenic, as mentioned before. When compared with the titrimetric procedures available for the measurement of cacodylate, the sensitivity of the differential pulse polarographic method is substantially greater and permits differentiation of organically bonded and inorganic arsenic.

It is recommended that the proposed differential pulse polarographic method for the analysis of cacodylate injections be subjected to an interlaboratory collaborative study for possible adoption by the AOAC as an official first action method.

This report of the Associate Referee was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Acknowledgment

The author wishes to express his appreciation to Thomas Medwick, Science Advisor, Food and Drug Administration, New York District, and Professor of Pharmaceutical Chemistry, Rutgers University, New Brunswick, NJ, for his invaluable assistance in the preparation of this paper.

REFERENCES

(1) *National Formulary* (1955) 10th Ed., American Pharmaceutical Association, Washington, DC, pp. 529–530
(2) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, sec. 36.043
(3) Holak, W. (1972) *J. Pharm. Sci.* **61**, 1635–1638
(4) Myers, D. J., & Osteryoung, J. (1973) *Anal. Chem.* **45**, 267–271
(5) Stone, L. R. (1967) *JAOAC* **50**, 1361–1362
(6) Sandell, E. B. (1959) *Colorimetric Determination of Traces of Metals*, 3rd Ed., Interscience Publishers, Inc., New York, NY, p. 278

Infrared Spectrophotometric Analysis of Medicinal Gases for Trace Impurities

WILSON L. BRANNON, WALTER R. BENSON, and GEORGE SCHWARTZMAN*
Division of Drug Chemistry, Food and Drug Administration, Washington, DC 20204
** Pharmaceutical Research and Testing, Food and Drug Administration, Washington, DC 20204*

The feasibility of examining medicinal gases for trace impurities, using an infrared spectrophotometer in conjunction with a 10 m gas cell, was investigated. Many of the impurities for which the USP includes limits were detected and measured at concentrations at or below those permitted by the USP; these include sulfur dioxide, carbon dioxide, carbon monoxide, and water. However, others (hydrogen sulfide, nitric oxide, and nitrogen dioxide) were not detected at these levels by this technique. Methane was found at low levels in some samples.

Numerous methods used in the analysis for pollutants in air employ infrared (IR) spectrophotometry (1). This laboratory has explored the applicability of this technique to the analysis of contaminants in medicinal gases for which limits are set in the USP (2); *see* Table 1. Using a 10 m multipath gas cell fitted to an IR spectrophotometer, a number of standard gas mixtures of nitrogen to which known amounts of contaminants had been added at levels approximating the USP limits (2) were examined. The method developed was found to be suitable for detecting and measuring the concentrations of carbon monoxide, sulfur dioxide, water, and carbon dioxide at these levels but it is not, however, applicable for the determination of hydrogen sulfide, nitric oxide, nitrogen dioxide, or oxygen as contaminants.

The IR technique described has several advantages over the use of the gas detector tubes specified in the USP methods: The IR technique is simpler because all IR-absorbing gaseous compounds can be detected in one spectrum if their concentrations are high enough; the IR technique requires only one cell, with a fixed volume, and is specific; and the IR technique measures the compound directly, rather than a reaction product.

METHOD

Apparatus and Reagents

(a) *Gas cell.*—10 m multipath (Perkin-Elmer Corp., Main Ave, Norwalk, CT 06852, No. 186-0214), modified as shown in Fig. 1, and instruction manual (Perkin-Elmer Corp., No. 990-9564).

(b) *Grating infrared spectrophotometer.* — Model 621 (Perkin-Elmer Corp.).

(c) *Pumps.*—(*1*) *Vacuum pump.*—½ horsepower (Fisher Scientific Co., 7722 Fenton St, Silver Spring, MD 20910, Welch Duo-seal). (*2*) *Com-*

Table 1. USP limits of gas impurities, ppm

Gas	Impurity						
	SO_2	H_2S	CO	NO, NO_2	CO_2	H_2O	Other
Nitrogen[a]	none	none	10[b]	none	300[b]	35.7[b]	odors, oxygen 10,000
Oxygen	none	none	10[c]	5[d]	300[c]	31.2[b]	odors
Carbon dioxide	5	1[e]	10	2.5 (NO) 2.5 (NO_2)	none	3[f]	ammonia 25
Helium	none	none	10				odor, halogens 1
Nitrous oxide	none	none	10	1 (NO) 1 (NO_2)	300	3	ammonia 25
Cyclopropane	none	none		none	300	none	halogens, propylene, allene

[a] Pharmaceutic aid.
[b] USP 18th (4), deleted from USP 19th (2).
[c] Exempt as in First Supplement (2), if produced by the air liquefaction process.
[d] Deleted as in First Supplement (2).
[e] No reliable, stable standard is available (E. Hughes, 1975, National Bureau of Standards, Washington, DC).
[f] mg/22 L.

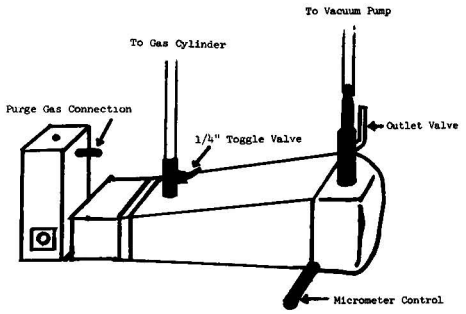


FIG. 1—Modified gas cell.

bination pressure-vacuum pump.—⅙ horsepower (Fisher Scientific Co.).

(d) *Adsorbents.*—Ascarite, 8–20 mesh (Arthur H. Thomas Co., Vine St, Philadelphia, PA 19105), used in 8 × 2″ glass column; and silica gel G, 16 mesh, grade 42 (Fisher Scientific Co., Tel-tale), used in 28 × 4″ column.

(e) *Gases.*—Standard gas mixtures of nitrogen with contaminants at concentrations close to the USP limits and commercial samples of medicinal gases (purchased on open market); *see* Table 2.

(f) *Toggle valve.*—¼″ (*see* Fig. 1) (Potomac Valve and Fitting, Inc., 12601 Twinbrook Pkwy, Rockville, MD 20852, Whitey SS-1GM4).

Determination

Blank spectrum.—Purge IR spectrophotometer and gas cell transfer optics (except the cell body) according to manufacturer's instructions with air that has been forced through 2 columns of ascarite and 1 column of silica gel by means of small pump at rate of ca 10 cu ft/hr. Purge ≥1 hr. Maintain purge while attaching tank containing sample gas to gas cell by means of port, stainless steel tubing, and regulator. Decrease pressure inside gas cell and tubing to 1 mm with second pump, close valve leading to pump, and record blank spectrum. Examine blank spectrum for possible interferences.

Sample spectrum.—Introduce sample gas directly (not through purging system) into evacuated gas cell by opening regulator and gas cell valves. Obtain positive pressure of ca 1 atmosphere. Repeat this procedure. Close cell valve and disconnect vacuum pump. Let gas in cell come to room pressure by intermittently opening gas cell valve. Close this valve and obtain IR spectrum. Under same conditions, obtain standard gas IR spectrum. Compare these spectra to determine contaminants present.

Received March 11, 1976.

Table 2. Analysis of commercial standards by IR spectrophotometry

Standard[a,b]	Concn, ppm	Detected
1. Carbon dioxide	363	yes
2. Carbon dioxide	456	yes
3. Carbon monoxide	11.6	yes
4. Nitric oxide	6.2	no
5. Nitrogen dioxide	6.1 (7.4)[c]	no
6. Sulfur dioxide	7.2	yes
7. Hydrogen sulfide	4 (1.3)[d]	no
8. Nitric oxide	48	yes
9. Nitrogen dioxide[e]	47	yes
10. Hydrogen sulfide	44	no
11. Methane	9.9	yes
12. Water	25	yes

[a] These are gases prepared by companies at concentrations that approximate USP and NF limits except for methane: MG Scientific, Division of MG Technical Products, Inc., 1100 Harrison Ave, Kearney, NJ 07029, Nos. 1-7 and 12; Airco Industrial Gases Division, Airco Inc., 575 Mountain Ave, Murray Hill, NJ 07974, Nos. 8-11.
[b] Nitrogen is diluent except for No. 9.
[c] Re-analysis for nitrogen dioxide 169 days after initial delivery.
[d] Re-analysis for hydrogen sulfide 270 days after initial delivery.
[e] Air is diluent.

Results and Discussion

The medicinal gases in the USP (2, 3) are given in Table 1, along with the impurities for which limits are given in the same USP monograph. Standard gas mixtures of nitrogen with the contaminants at concentrations close to their USP limits were introduced into the IR gas cell as described above. Both the standards and the samples were measured under the same conditions. The carbon dioxide, water, carbon monoxide, and sulfur dioxide standards gave IR absorption bands suitable for showing compliance and noncompliance with the USP limits. Nitric oxide, nitrogen dioxide, and hydrogen sulfide were not detected at those levels. Nitric oxide and nitrogen dioxide could be detected at higher levels (*see* Table 2) but hydrogen sulfide was not detected, even when it was present at a concentration of about 50 times the USP limit. Oxygen, as a contaminant of nitrogen, does not show IR absorption bands and cannot be detected.

Each contaminant has an absorption region which is characteristic of that gas, as shown in Fig. 2. All of the regions reported (4–7) for each gas contaminant are listed in Table 3, together with the regions found in this study. In general,

This paper was presented at the 89th Annual Meeting of the AOAC Oct. 13–17, 1975, at Washington, DC.

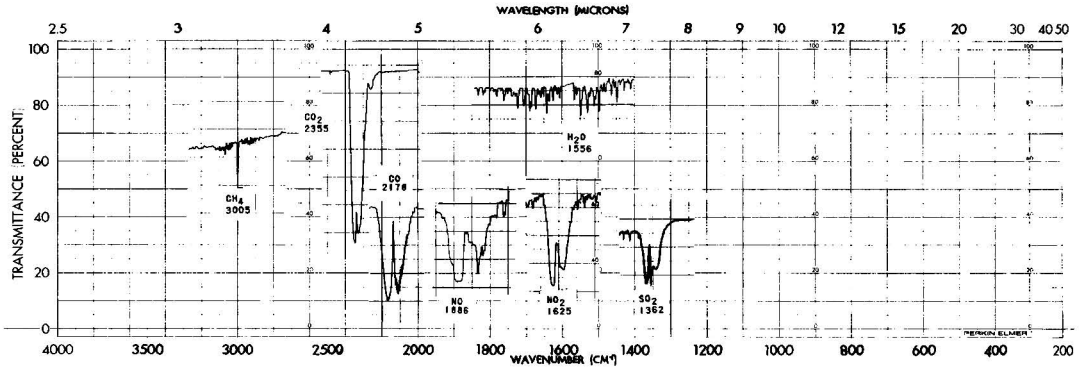


FIG. 2—IR absorption data for gases.

the IR region for each contaminant is different. The sulfur dioxide region appears at the edge of the water region. However, when the water concentration is 25 ppm, the sulfur dioxide absorption region can be measured at its USP limit without interference. The nitrogen dioxide region appears in the middle of a broad water region and these 2 components would interfere if they were present in the same sample. However, the water band has a broad, characteristic set of absorption peaks and can be identified behind the much narrower set of peaks in the nitrogen dioxide region. If nitrogen dioxide were present at its USP limit, a water concentration greater than 25 ppm would interfere with its measurement.

Ammonia was not examined with this system; however, it can be detected at the 25 ppm contaminant level in the 900–1000 cm^{-1} region (8). Two characteristic peaks for ammonia occur at about 930 and 965 cm^{-1} and a characteristic pattern is also clearly formed by the smaller bands. These 2 peaks do not interfere nor are they interfered with by the IR absorptions of the other contaminants given in Table 1.

Table 3. IR absorption data for gases

Gas	*A* (7, 8), L, cm^{-1}	*A* (this lab.,) cm^{-1}	Range,[a] cm^{-1}
Hydrogen sulfide	1290	1289[b]	1100–1420
Carbon monoxide	2176 (7), 2140 (8)	2178	
Sulfur dioxide	1361	1362	
Nitric oxide	1875	1886	1750–1950
Nitrogen dioxide	1618	1625	
Water	1590	1556	1330–1880
Carbon dioxide	2320	2355	
Methane	3000	3005	

[a] In some cases the band shape is subject to change so a range is used.

[b] Hydrogen sulfide was not observed at 44 ppm but this band was found at higher concentrations.

The IR measurements of the contaminants in nitrogen, using a 10 m gas cell with no expansion, gave the following approximate detection limits (in ppm): nitrogen dioxide 7, sulfur dioxide 2, methane 10, carbon dioxide 43, and water 5. With a 10× ordinate scale expansion, the IR detection limit measurements for nitric oxide and carbon monoxide were 6 and 4, respectively.

Eleven samples of various medicinal gases (Table 4), were analyzed by the proposed method. In general, the contaminant gases listed in Table 1 were detected at levels lower than the USP limits.

In the IR spectrum of all samples of oxygen alone or admixed with other gases, unexpected peaks appeared at 3000 and 1308 cm^{-1}. These 2 absorption frequencies are characteristic for methane. In order to see if there were other gases which could give similar absorbances, a computer-assisted search of IR data (L. R. Dusold, 1976, Food and Drug Administration, Washington, DC, private communication) involving over 100,000 compounds and mixtures of compounds was initiated. The search eliminated several possible compounds which exhibited either an additional band or a shift in the position of the related bands and confirmed the initial identification as methane.

Methane at levels of approximately 20–40 ppm was found in 3 samples of USP oxygen.

Table 4. Comparison of sample and standard IR absorptions[a]

Sample[b]	Standards, ppm								
	SO_2	NO[c]	NO_2	H_2O	CO[c]	CO_2	CO_2	H_2S	CH_4
Standard, ppm	7.2	48	47	25	11.6	36.3	45.0	44	9.9
1. 93% O_2 7% CO_2	L[d]	L	L	L	L	M[e]	M	ND[f]	M
2. 5% CO_2 95% Air	L	L	L	L	L	M	M	ND	L
3. Lung diffusion	L	L	L	L	M	L	L	ND	M
4. USP O_2 No. 200	L	L	L	L	L	L	L	ND	M
5. USP O_2 360L	L	L	L	L	L	L	L	ND	M
6. USP O_2 625L	L	L	L	L	L	L	L	ND	M
7. Breathing air	L	L	L	L	L	L	L	ND	L
8. 70% O_2 30% CO_2	L	L	L	L	L	M	M	ND	M
9. Nitrous oxide	L	L	L	L	I[g]	I	I	ND	L
10. Cyclopropane	I	I	I	I	I	I	I	I	I
11. Helium	L	L	L	L	L	L	L	L	L

[a] Data were obtained by using 1× ordinate expansion.
[b] Sample Nos. 1, 2, 4–9, and 11 were obtained from Airco, Inc.; Sample Nos. 3 and 10 were obtained from MG Scientific.
[c] Data were obtained by using 10× ordinate expansion.
[d] Sample has less (L) IR absorption than standard or was not detectable at limit of method.
[e] Sample has more (M) IR absorption than standard.
[f] ND = no data obtained at that concentration because standard does not show IR absorbance in this system.
[g] I = IR absorption of sample interferes with absorbance of standard.

Although the USP monograph includes no limits on the presence of methane, its possible presence is recognized by the U.S. Navy which sets a limit of 25 ppm methane in aviator-breathing oxygen (9). A recommendation to the USP for inclusion of this limit, using the method described to determine the methane content, will be made.

No sorption on the walls or the rubber O-rings of the gas cell was encountered for carbon monoxide and dioxide, sulfur dioxide, methane, nitric oxide, and water. However, when the cell was used for the examination of other gases, e.g., nitrogen dioxide, nitrous oxide, and cyclopropane, these gases were probably sorbed on the cell walls, etc. The spectrum of the empty gas cell did not show their presence after initial use with proper evacuation but, on standing, these gases desorbed and affected the spectra of subsequent samples. Several days were required to free the cell of these gases. Thus, care must be exercised when dealing with gases other than those shown not to sorb.

Some gases may react with the KBr window. When nitrogen dioxide was left in the cell for 1 hr, its characteristic IR absorption region began to disappear and another band appeared. This new band was characteristic of a nitrate salt (10). Polishing the KBr windows removed this IR band. Although the lower limit of detectability of nitrogen dioxide could be improved by the use of a 10× ordinate scale expansion, which would bring the sensitivity below the limit given by the USP (Table 1), the problems of reactivity with the KBr window and possibly other surrounding material, sorption in the tank used for the standard (*see* Table 2), and sorption in the chamber led us to conclude that this method is not reliable for the determination of nitrogen dioxide. In addition, although the National Bureau of Standards has a standard reference material which will generate a gas containing 50 ppm nitrogen dioxide, it does not provide standard reference materials which generate stable standards at the lower concentrations needed for the USP limits.

The method is not completely satisfactory for several other gases because of interferences in the IR absorption regions, as shown in Table 4. For example, nitrous oxide interferes with the absorption bands of carbon monoxide and carbon dioxide, but some open areas remain in the spectrum so that methane, sulfur dioxide, and water can still be identified and quantitatively measured. Cyclopropane, on the other hand, absorbs in all of the IR regions of interest. Nitric oxide and nitrogen dioxide cannot be detected at the low levels specified in the USP limits.

Hydrogen sulfide was not detectable, even at very high concentrations.

The method described is a simple, rapid, specific, and quantitative method for trace amounts of water, carbon dioxide, carbon monoxide, sulfur dioxide, and methane and also a variety of IR-absorbing gaseous contaminant compounds (11), such as volatile hydrocarbons, halogenated hydrocarbons (e.g., methyl chloride, chloroform, carbon tetrachloride, freons, and halogenated anesthetics), ethers, ketones, and esters. However, the analyst must be aware of the possibility of sorption, as was reported earlier for methyl methacrylate (12). The application of this general technique resulted in finding methane and this presents a strong argument for the use of IR in screening medicinal gases for possible contaminants not listed in their specifications.

References

(1) Saltzman, B. E., & Cuddebach, J. E. (1975) *Anal. Chem.* **47**(5), 1R-15R

(2) *United States Pharmacopeia* (1975) 19th Rev. and 1st Supplement (1976) Mack Publishing Co., Easton, PA

(3) *United States Pharmacopeia* (1970) 18th Rev., Mack Publishing Co., Easton, PA

(4) Hanst, P. L., Lefohn, A. S., & Gay, B. W., Jr. (1973) *Appl. Spectrosc.* **27**, 188–198

(5) Zeller, M. V., & Juszli, M. P. (1973) Infrared Bull. No. 36, Perkin-Elmer Corp., Norwalk, CT

(6) Kemmner, J. G., Nonnenmacher, G., & Wehling, W. (1966) *Fresenius Z. Anal. Chem.* **222**, 149–161; thru *Chem. Abstr.* (1967) **66** 25734

(7) Campani, P., Fang, C. S., & Prengle, H. W., Jr. (1972) *Appl. Spectrosc.* **26**, 372–381

(8) *The Sadtler Standard Spectra* (1959) Sadtler Research Laboratories, Philadelphia, PA, Spectrum No. 93

(9) Kagarise, R. E., & Saunders, R. A. (1960) Nav. Res. Rep. No. 5554, U.S. Naval Research Laboratory, Washington, DC

(10) Zeller, M. V. (1974) Pittsburgh Analytical Conference, Cleveland, OH, March 4–8, Paper No. 360

(11) Industrial Product Data Sheet No. IR-7624 (Aug. 1974), Beckman Instruments, Inc., Fullerton, CA

(12) Sheinin, E. B., Benson, W. R., & Brannon, W. L. (1976) *J. Pharm. Sci.* **65**, 280–283

Pilocarpine Hydrochloride in Ophthalmic Solutions: Modification of a High-Pressure Liquid Chromatographic Determination and Survey

JOHN D. WEBER
Division of Drug Chemistry, Food and Drug Administration, Washington, DC 20204

Pilocarpine undergoes 2 important reactions in solutions, epimerization to isopilocarpine and hydrolysis to pilocarpic acid. There is no official USP limit for isopilocarpine or pilocarpic acid in pilocarpine hydrochloride ophthalmic solutions. An existing high-pressure liquid chromatographic (HPLC) method was modified by changing the buffer system to permit its use with constant-pressure as well as constant-volume instruments. The procedure separates all 3 components on a cation exchange resin; pilocarpine and isopilocarpine are determined directly and pilocarpic acid indirectly. Ophthalmic solutions and drug substances were obtained from substantially all the United States marketers of pilocarpine opthalmic solutions and were analyzed by the modified HPLC method. Results of the survey show that isopilocarpine is present to the extent of 0.4–3% and pilocarpic acid at levels of 0.6–7% of total alkaloid.

Pilocarpine is used as a miotic in the treatment of glaucoma and retinal detachment. It undergoes 2 important reactions in solutions, epimerization to isopilocarpine and hydrolysis to pilocarpic acid. Both reactions are base-catalyzed; the latter is reversible, while the former is not. Both reactions result in the loss of pharmacological activity. The history, pharmacology, chemistry, and stereochemistry of pilocarpine are briefly reviewed with pertinent references in the literature to the assay of pilocarpine (1–4). While the epimer, isopilocarpine, lacks the pharmacological activity of pilocarpine (5), there is no official limit *per se* for isopilocarpine or pilocarpic acid in pilocarpine hydrochloride or nitrate (6). The USP specifies a specific rotation of +88.5 to +91.0° for pilocarpine hydrochloride (6). That range permits up to 6% isopilocarpine or 3% pilocarpic acid contamination. The official assay, based upon the formation of the hydroxamic acid (7), determines both pilocarpine and isopilocarpine (4) but does not detect pilocarpic acid (7). Urbányi *et al.* (4) demonstrated this experimentally and reported the development of an elegant method for the simultaneous determination of both isopilocarpine and pilocarpine in ophthalmic solutions by high-pressure liquid chromatography (HPLC) on an ion exchange resin column.

Previously it had been clearly demonstrated that the epimerization of pilocarpine occurs in solutions of pH 6.5–7.0 (8, 9). In another investigation, the reversible hydrolysis of pilocarpine to pilocarpic acid was examined (2). None of these studies could be correlated with the situation which exists with marketed ophthalmic solutions, although several authors (2, 8, 9) claim, without documentation, that it is well known that there is no problem of stability with solutions of pH 3.5–5.5, the pH range of official USP solutions (6).

The Food and Drug Administration (FDA) initiated a nationwide survey of marketed pilocarpine hydrochloride ophthalmic solutions. A random sampling was obtained of one or more products from 10 manufacturers and packagers of pilocarpine hydrochloride ophthalmic solutions, substantially all those in the United States. In addition, FDA inspectors obtained samples of bulk drug substances from the manufacturer or packager. The samples were subdivided and one portion was analyzed by the official USP method in an FDA District laboratory for compliance with existing USP requirements. The other portion was sent to the Associate Referee's laboratory for the determination of isopilocarpine and pilocarpic acid.

Prior to the receipt of the samples an effort was made to either locate a published method or develop a suitable analytical method. When this investigation was initiated late in 1973, only one literature report of the analytical separation of pilocarpine and isopilocarpine was found (10), a questionable separation by thin layer chromatography (TLC). The reported separation of the epimers could not be duplicated in this laboratory, using the 2 TLC solvent

systems given (10), as well as over 100 reasonable variations. A comprehensive 1974 study (3) of the epimerization and hydrolysis of pilocarpine described the use of nuclear magnetic resonance (NMR) spectrometry at 100 MHz to quantitate the 2 epimers. This laboratory does not have a 100 MHz NMR spectrometer and the 2 epimers could not be distinguished by NMR at 60 MHz (11, 12).

Since the epimers have clearly different infrared (IR) spectra, an effort was made to analyze epimeric mixtures by IR. However, the epimeric composition of pilocarpine and isopilocarpine mixtures could not be determined by using the differences in their IR spectra, unless the epimers were of about equal concentrations.

Since polarimetry has been used to determine the epimeric composition of pilocarpine solutions (2, 9), the Associate Referee developed a method by which epimeric composition was calculated from polarimetric measurements and colorimetric determination of total alkaloids subsequent to preliminary chromatographic separation of pilocarpic acid. The method was successful for standard solutions of pilocarpine, isopilocarpine, and pilocarpic acid generated *in situ* as well as for simulated samples which contained typical thickeners, buffers, and preservatives. The method was capable of supplying the data needed in this study; however, a major drawback was the amount of effort and time needed for each determination.

At this point the HPLC method of Urbányi *et al.* (4) was received for pre-publication review. In evaluating the applicability of their HPLC procedure to this study, it was necessary to change the buffer because of a salient difference between our laboratory-assembled high-pressure liquid chromatograph and their instrument. The difference, which is also common to some commercial instruments, involves the type of eluant pump. Their instrument (4) has a constant-volume pump, adjusting its pressure generation to that necessary to maintain the selected flow rate, while our laboratory-assembled instrument, along with a number of commercial instruments, has a constant-pressure eluant pump. The HPLC method of Urbányi *et al.* (4) has been used to determine the isomeric purity of radiolabeled pilocarpine (13).

Received October 29, 1975.

METHOD

Reagents

(a) *Mobile phase.*—0.1*M* Na_2HPO_4 in 5% isopropanol. Dissolve 28.2 g anhydrous reagent grade Na_2HPO_4 in ca 800 ml water, add 50 ml isopropanol, and dilute to 1 L with water. Adjust pH to 9.0±0.05 by dropwise addition of dilute H_3PO_4 (3+7). Filter solution through 5 μm pore filter (No. LS 47, Millipore Corp., Bedford, MA), in 250 ml filter holder (No. XX10 047 03), or equivalent.

(b) *Sodium sulfate solution.*—0.2*M*. Dissolve 28.4 g reagent grade anhydrous Na_2SO_4 in water and dilute to 1 L with water.

(c) *Cation exchange resin.*—Aminex A-7, sodium form, 9±2 μm beads (Bio-Rad Laboratories, Richmond, CA).

(d) *Sulfuric acid.*—1*N*. Carefully add 28 ml concentrated H_2SO_4 to ca 800 ml water, cool, and dilute to 1 L.

(e) *Trisodium phosphate solution.*—0.5*M*. Dissolve 19.0 g reagent grade $Na_3PO_4 \cdot 12H_2O$ in water and dilute to 1 L.

(f) *Pilocarpine standard solution.*—Dissolve ca 15 mg, accurately weighed, USP Reference Standard Pilocarpine Nitrate in 0.2*M* Na_2SO_4 and dilute to 25.0 ml to give final concentration of ca 0.6 mg/ml.

(g) *Pilocarpine-isopilocarpine test solution.*—Dissolve ca 15 mg, accurately weighed, isopilocarpine nitrate (Aldrich Chemical Co., Metuchen, NJ) in 0.2*M* Na_2SO_4 and dilute to 100 ml with 0.2*M* Na_2SO_4. Dissolve ca 15 mg, accurately weighed, USP Reference Standard Pilocarpine Nitrate in 0.2*M* Na_2SO_4, add 1.0 ml isopilocarpine nitrate solution, and dilute to 25.0 ml.

Apparatus

(a) *High-pressure liquid chromatograph.*—With ultraviolet (UV) detector capable of measurement at 216 nm, cell of 10 mm pathlength and ca 10 μl volume, and integrator to determine peak area. (A larger cell of 1 mm pathlength and 25 μl volume is satisfactory but there is less resolution.)

(b) *Column.*—Thin wall 0.25″ od stainless steel tube, 65 mm × 5.5 mm, fitted at outlet with stainless steel frit with 2 μm pores. (A 100 mm × 4 mm id tube is also suitable, but has longer retention times; the 100 mm × 6 mm id glass tube used originally (4) is also satisfactory.) Suspend ca 4 ml dry resin in 25 ml mobile phase and slurry-pack column at ca 100 psig. Cap column with stainless steel frit with 2 μm pores. Alternatively, if pressurized slurry column-packer is not available, apply vacuum to column outlet and add dropwise suspension of 4 ml dry resin in 10 ml

mobile phase. Mount column in instrument and condition by eluting with mobile phase 2 hr at 0.6 ml/min. Remove column and fill any void at top with resin slurry as before.

Preparation of Samples

(a) *For intact pilocarpine and isopilocarpine.*—Dilute volume of ophthalmic solution containing ca 15 mg pilocarpine hydrochloride to 25.0 ml with 0.2*M* Na_2SO_4.

(b) *For total alkaloids.*—Transfer same volume of ophthalmic solution used in (a) to volumetric flask; add 3 ml 1*N* H_2SO_4, and let stand 2 hr. Add 2 ml 0.5*M* trisodium phosphate solution and dilute to volume with 0.2*M* Na_2SO_4.

Determination

Fit column in instrument and condition by eluting with mobile phase at 0.6 ml/min until stable baseline is obtained, measuring absorbance at 216 nm.

Inject 10 μl pilocarpine-isopilocarpine test solution and adjust sensitivity so that pilocarpine peak is ca 60% full scale. The method of sample introduction depends on equipment available on chromatograph. A measured sampling loop, an injection loop, and an injection block were all satisfactory. A sampling loop of measured volume was used in the survey.

Inject and record chromatograms, sequentially, of duplicate 10 μl injections of sample preparation (a), single 10 μl injection of pilocarpine standard solution, and duplicate 10 μl injections of sample preparation (b).

Calculations

Determine peak areas for isopilocarpine and pilocarpine for each injection. If isopilocarpine peak is present on chromatogram of the standard solution, use sum of both peaks as standard area.

(a) *Pilocarpine content of sample.*

$$\text{Pilocarpine salt in sample, mg/ml} = (C/V) \times F \times [P/(\text{i-}P' + P')]$$

where C = concentration of pilocarpine standard solution, in mg/ml; V = ml sample taken; P and P' = peak areas due to pilocarpine in sample preparation (a) and standard solution, respectively; i-P' = peak area due to isopilocarpine in standard solution (if present); and F = factor for dilution and molecular weight (22.55 for pilocarpine hydrochloride ophthalmic solutions and 25 for pilocarpine nitrate ophthalmic solutions).

(b) *Isopilocarpine content of sample.*

$$\text{Isopilocarpine, \% of total alkaloid} = [\text{i-}P/(\text{i-}P + P)] \times 100$$

where i-P = peak area due to isopilocarpine sample preparation (a).

(c) *Pilocarpic acid.*

$$\text{Pilocarpic acid, mg/ml} = P_b - P_a$$

where P_a and P_b = pilocarpine content calculated from sample preparations (a) and (b), respectively.

Results and Discussion

Method Development

As noted before, the method of Urbányi *et al.* (4) is not suitable for a high-pressure liquid chromatograph equipped with a constant-pressure eluant pump. The problem manifested itself as an increase in retention time with each subsequent injection; *see* Fig. 1. The potential for this problem had been observed (4) as a gradual rise in operating pressure with time. An apparatus with a constant pressure eluant supply, which does not compensate for the increased pressure drop across the column, and the *tris*-(hydroxymethyl)methylamine (trisamine) buffer recommended (4) was used initially. The retention time at peak maximum for pilocarpine was initially about 15 min on a 65 mm × 5.5 mm id column at a constant 140 psig and it increased by about 4 min with each subsequent injection. The peaks broadened until they disappeared in the baseline noise (injection 5, Fig. 1). The increase in retention times was found to be a function of time of exposure of the resin to the buffer, rather than the number of injections. The flow rate decreased simultaneously, from an initial value of about 0.5 ml/min to about 0.1 ml/min. A microscopic examination of the resin beads showed no change in shape, size, or birefringence before and after use. In an effort to ascertain the cause of the drop in flow rate, a column was emptied, the resin was washed and decanted several times, and the column was repacked with the "spent" resin. The flow rate increased to that of a new resin column, but retention times remained long. It was hypothesized that the problem was due to permeation of the resin beads with the large *tris*-(hydroxymethyl)methylammonium ion in the buffer, "exposing" additional internal sulfonate groups and effectively increasing the number of exchange sites. If that were the case, the use of a buffer with the less bulky sodium cation would prevent permeation, since the resin is supplied in the sodium form. An 0.1*M* Na_2HPO_4 buffer

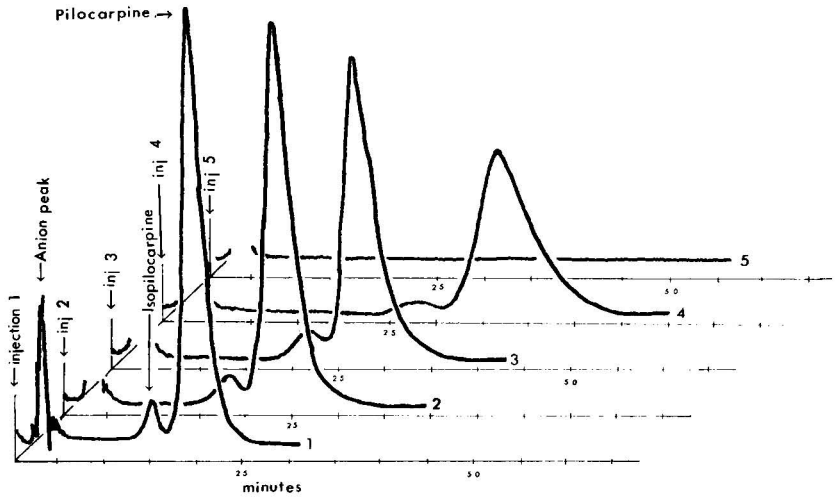


FIG. 1—Five successive injections of pilocarpine hydrochloride containing 5% isopilocarpine. Injecton 5 was made about 10 hr after injection 4 and peaks are lost in baseline noise. Eluant was the pH 9 trisamine buffer of Urbányi *et al.* (4). Anion peak deleted from tracings 2–5.

was substituted for the 0.2*M* trisamine buffer as the eluting solvent. Using this, only an insignificant increase in retention time was seen after 150 injections; at this time the column failed due to an accumulation of carboxymethyl cellulose gel from the sample on top of the resin bed. This simple expedient extends the very useful method of Urbányi *et al.* (4) to high-pressure liquid chromatographs equipped with constant-pressure pumps.

The second problem encountered resulted from the modification. Apparently pilocarpine undergoes specific anion-catalyzed hydrolysis (8, 10, 14) and, as a result, is hydrolyzed at an unacceptably high rate in pH 9 phosphate buffer, (*see* Fig. 2) while it is more stable in the pH 9 trisamine buffer specified by Urbányi *et al.* For that reason 0.2*M* Na_2SO_4 is used for preparing the standard and sample solutions instead of the mobile phase. Using this electrolyte, there was no measurable hydrolysis or epimerization of pilocarpine, even 3 days after dilution. Water, of course, does not hydrolyze pilocarpine, but with water as the diluent, there is a serious disturbance in the baseline of the chromatogram.

Urbányi *et al.* (4) were able to detect 25 ppm isopilocarpine, which is more than adequate for all commercial samples examined. The peaks were qualitatively identical to those reported (4). Injection 1 in Fig. 1 is representative. At pH 9, pilocarpic acid is eluted with other anions; therefore, the pilocarpic acid content could be calculated from the area of the anion peak on the chromatogram if no other UV-absorbing anions are present. The area of the anion peak is multiplied by an experimentally determined factor of 0.81 to obtain the equiva-

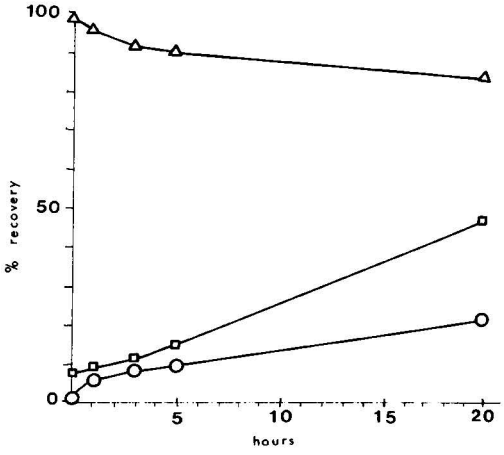


FIG. 2—Hydrolysis of pilocarpine in pH 9 phosphate buffer at 25°C: △, pilocarpine; ○, pilocarpic acid; □, isopilocarpine, per cent × 10.

Table 1. Determination of pilocarpine, isopilocarpine, and pilocarpic acid in ophthalmic solutions of pilocarpine hydrochloride

Sample[a]	Pilocarpine			Isopilocarpine, % of total alkaloids	Pilocarpic acid[c], % of total alkaloids	pH of solution
	Found, %[b]	Declared, %	% of declared found			
1	0.476	0.5	95.2	0.95	4.6	4.25
2	0.963	1.0	96.3	3.4	6.8	5.05
3	0.520	0.5	104.0	1.98	0.6	4.98
4	1.00	1.0	100.0	2.5	4.0	5.20
5	0.97	1.0	97.0	0.4	6.0	3.72
6	0.516	0.5	103.2	1.5	4.8	4.55
7	2.93	3.0	97.7	0.63	6.3	3.55
8	0.255	0.25	102.0	2.62	4.0	5.00
9	1.39	1.5	92.7	1.4	2.0	4.00
10	2.00	2.0	100.0	0.7	3.0	4.45
11	1.02	1.0	102.0	0.7	4.0	3.60
12	0.937	1.0	93.7	1.53	0.6	3.95

[a] Samples 2-4 are from the same manufacturer; the rest are from different manufacturers.
[b] Average of 2 determinations.
[c] Difference between average of 2 determinations, each with and without acid pretreatment; see *Method*.

lent of pilocarpine. With ophthalmic solutions, unknown anions in the buffer, thickening agents, and some preservatives prevent direct determination of pilocarpic acid. When these interfering substances are present, pilocarpic acid may be determined by recyclization to pilocarpine in sulfuric acid; the increase in pilocarpine in the sample thus treated is used to calculate pilocarpic acid.

An aliquot of the sample is mixed with an equal volume of 1*N* H_2SO_4 and allowed to stand for 2 hr; the solution is then partially neutralized with 0.5*M* Na_3PO_4 solution as described under *Method*, diluted, and chromatographed. The pilocarpine content found in the assay is subtracted from the pilocarpine content determined after the acid recyclization; the additional pilocarpine represents the pilocarpic acid in the sample. At the lower levels, the pilocarpic acid content is just larger than the uncertainty in the measurement, estimated to be ±1% of total alkaloids. Pilocarpic acid content with values less than 2% of total alkaloids are only estimates.

Pilocarpic acid and isopilocarpic acid are not distinguishable by the difference method. However, it is apparent that isopilocarpic acid never represented more than a few per cent of the combined pilocarpic acid-isopilocarpic acid content, since isopilocarpic acid is recyclized to isopilocarpine and a disproportionate increase in isopilocarpine content after the recyclization was never observed.

Results of the Survey

The samples were analyzed for pilocarpine, isopilocarpine, and pilocarpic acid; *see* Table 1. Since the quantities were related to peak areas, not peak heights, and since the instrument used did not have an integrator, relative peak areas were determined by weighing cut-outs of Xerox copies of the peaks. Simultaneously a control, a 4 × 4″ square of each copy page of the individual chromatograms (i.e., one control square for each injection), was weighed. The average weight of 47 test squares was 199.9±4.85 mg. An integrator would certainly have given better precision. There were 5 injections for each sample, 2 diluted samples, 2 acid-treated samples, and 1 standard. Isopilocarpine was calculated by using pilocarpine as an internal standard, and pilocarpine was calculated from injection of pilocarpine standard.

The standard contained about 1% isopilocarpine; results of 14 determinations were 0.96±0.2% isopilocarpine as per cent of total alkaloid, which is indicative that the precision of the method of analysis is better than the precision of the method of quantitation.

A correlation between isopilocarpine content and pH is observed, which is too high to be coincidental. A least square fit of isopilocarpine (as per cent of total alkaloid) vs. pH of solution gave a slope of 0.54 and an intercept at pH 3.5 with a correlation coefficient of 0.84 (*see* Fig. 3). When the diverse sources of these solutions are taken into consideration, 0.84 is a rather good

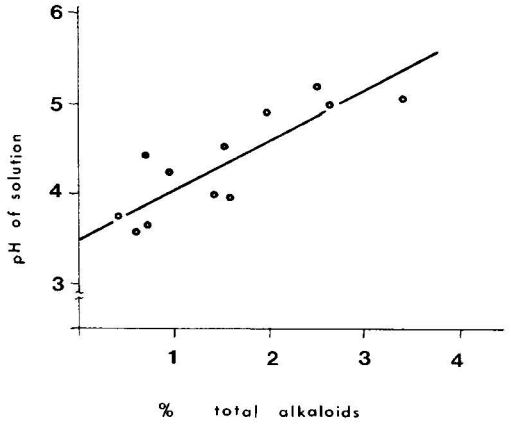


FIG. 3—Correlation of isopilocarpine content (as per cent of total alkaloids) and pH of ophthalmic solution. The circles are experimental values and the line is least square fit with all points equally weighted.

correlation coefficient. The value of the intercept seems to imply that 3.5 is the lower limit of pH for which epimerization will occur. Lest too much importance be placed on this observation, a reservation must be made: Urbányi *et al.* (4) reported values for the pH and isopilocarpine content of 7 commercial ophthalmic solutions and their data exhibited no simple correlation between pH and isopilocarpine content.

The pilocarpic acid contents found for the marketed solutions, listed in Table 1, ranged from 0.6 to 6.8% of total alkaloid, but the amounts showed no relationship to the pH of the solutions. Urbányi *et al.* (4) did not report having determined pilocarpic acid.

These two facts, the apparent pH dependence of isopilocarpine content and the apparent pH independence of pilocarpic acid content, are surprising. The epimerization of pilocarpine is reported (3) to be irreversible, or at least the equilibrium lies overwhelmingly in the direction of the *trans*-isomer, isopilocarpine. Based on that report (3) and other observations (2, 5, 8, 9) on the epimerization of pilocarpine, the isopilocarpine content of samples in the pH 3.5 to 5.5 range should depend only on the history of the sample, i.e., the method of sterilization, heating, storage conditions, buffer, and initial isopilocarpine content of the drug used to prepare the solution. On the other hand, the hydrolysis of pilocarpine is the reverse of the cyclization of pilocarpic acid, as shown below.

$$P \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} PA$$

The former is base-catalyzed and the latter is acid-catalyzed. At pH >6, the rate of hydrolysis (apparent first order rate constant) increases linearly with the pH, and at pH <3 the rate of cyclization increases linearly with a decrease in pH (2). If one combines Figs. 1 and 7 from ref. 2, a plot of rate of hydrolysis vs. pH and rate of cyclization vs. pH, respectively, the 2 curves should merge at a minimum value, expected between pH 3 and 8. Along this minimum, a series of equilibria should be established for which pilocarpic acid content should be a function of pH only. Actually, such an equilibrium has been reported (14) at pH 6.0, the equilibrium mixture containing 63% pilocarpine-isopilocarpine and 37% pilocarpic acid-isopilocarpic acid.

The probable reasons for the lack of relationship beween pH and pilocarpic acid content in the solutions in this survey are: (*1*) The initial pilocarpic acid content depends on the history of the sample (2), e.g., heating and buffer content, and (*2*) the rates of the reactions which would reestablish the equilibrium are exceedingly slow between pH 3 and 5 (2, 3, 8, 9) at room temperature and below, the normal storage conditions for ophthalmic solutions. The rate of hydrolysis is affected by the specific ion catalysis (8, 9, 14) but the position of the equilibrium is unaltered (14). It has been reported (14) that, at pH 6.0, although the rate at which the equilibrium is established varies with "gegen" ion, the equilibrium does not.

Of 13 samples of drug substance, all of which conformed to USP requirements (6), 3 were found to contain isopilocarpine, 0.60, 1.90, and 1.23% of total alkaloids, respectively.

Conclusions and Recommendations

All of the ophthalmic solutions examined conformed to USP XIX specifications (6) for pilo-

This report of the Associate Referee was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee B and were accepted by the Association. *See* (1976) *JAOAC* **59**, 382.

carpine content and pH. There is no USP limit for isopilocarpine or pilocarpic acid. All of the samples of pilocarpine hydrochloride drug substances examined in this survey met USP XIX specifications (6) with respect to melting range and optical rotation. Nonetheless readily measurable amounts of isopilocarpine were found in some of the samples.

As a result of the experience gained with the method during this survey, it is recommended that the modified HPLC assay for pilocarpine and isopilocarpine be collaboratively studied.

The principle defect in the determination of pilocarpic acid stems from the fact that one is determining a small difference between 2 relatively large values. The matter of an assay for pilocarpic acid should be subjected to further study.

Note Added in Proof.—The method was tested collaboratively and failed. Collaborators reported poor separations and unreasonably long retention times. The cause of the failure has not been ascertained.

References

(1) Murray, J. B. (1970) *Proc. Soc. Anal. Chem.* 107–109

(2) Chung, P.-H., Chin, T.-F., & Lach, J. L. (1970) *J. Pharm. Sci.* **59**, 1300–1306

(3) Nunes, M. A., & Brochmann-Hanssen, E. (1974) *J. Pharm. Sci.* **63**, 716–721

(4) Urbányi, T., Piedmont, A., Willis, E., & Manning, G. (1976) *J. Pharm. Sci.* **65**, 257–260

(5) Anderson, R. A., & Cowle, F. B. (1968) *Brit. J. Ophthalmol.* **52**, 607–611

(6) *United States Pharmacopeia* (1975) 19th Rev., Mack Publishing Co., Easton, PA, pp. 384–386

(7) Brochmann-Hanssen, E., Schmid, P., & Benmann, J. D. (1965) *J. Pharm. Sci.* **54**, 783–784

(8) Anderson, R. A., & Fitzgerald, S. D. (1967) *Aust. J. Pharm.* **48** (Suppl. No. 57), s108–s109

(9) Anderson, R. A. (1967) *Can. J. Pharm. Sci.* **2**, 25–26

(10) Massa, V., Gal, F., Susplugas, P., & Maestre, G. (1970) *Trav. Soc. Pharm. Montpellier* **30**, 267–272

(11) Baeschlin, K., Etter, J.-C., & Moll, H. (1969) *Pharm. Acta Helv.* **44**, 301–308

(12) Link, H., & Bernauer, K. (1972) *Helv. Chim. Acta* **55**, 1053–1064

(13) DeGraw, J. I., Engstrom, J. S., & Willis, E. (1975) *J. Pharm. Sci.* **64**, 1700–1701

(14) Testa, B., & Etter, J.-C. (1975) *Can. J. Pharm. Sci.* **10**, 16–20

Thin Layer Chromatographic Identification of Some Sympathomimetic Amines

JITENDRA J. SHAH and RAMILA J. SHAH
Woodson-Tenent Laboratories, Inc., 345 Adams Ave, Memphis, TN 38101

Thin layer chromatographic behavior of some sympathomimetic amines in the presence of acids in neutral and organic solvent systems is reported. The sympathomimetic amines were dissolved in 0.1*N* HCl or ethanol and treated with bromocresol green or *p*-nitrobenzoyl chloride reagents on fiber sheets or precoated glass plates. Two-, 3-, and 4-component solvent systems were tested. Benzene-ethyl acetate gave 2 spots for each amine standard; the more polar spots were satisfactorily separated. Amines in pharmaceuticals were not separated by any solvent system tested.

Thin layer chromatography (TLC) is increasingly used to separate and identify pharmaceuticals in mixtures and also to quantitatively determine the composition. TLC is not limited by the choice of spray reagents, a factor in paper chromatography, because of the nature of the carrier material. In studies with paper chromatography, West (1) observed double spots with trichloroacetic acid and tryptamine derivatives of sympathomimetic amines; later, Beckett *et al.* (2, 3) showed that the spot formation and multiplication occurs with many bases in the presence of many acids. Robinson and Shepherd (4) considered that the additional amine spot formed in the presence of trichloroacetic acid results from the formation of loose complexes between the bases, acting as electron donors, and the trichloroacetic acid, acting as an electron acceptor by virtue of the marked electronegativity of the 3 chlorine atoms. Roberts (5) also observed the phenomenon of multiple spot formation in an attempt to examine some factors which were affecting the R_f values of sympathomimetic amines, but on paper chromatography only. Hydrochloric acid in the developing solvent converted some of the norepinephrine acid tartarate to norepinephrine hydrochloride. The presence or absence of multiple spots, according to Roberts and Broadly (6), depends on the chemical structure of each amine.

Studies of TLC (7–9) indicated that, just as in paper chromatography, it is possible to obtain 2 spots of an amine salt (or pure amine in the presence of one or more equivalents of acid) when a neutral or acidic solvent system is used with cellulose as the thin layer. The paper chromatographic separation of adrenaline and noradrenaline, also sympathomimetic amines, has been described in some publications (10, 11) and ephedrine has been included in a chromatographic study of alkaloids (12). James (10) has also chromatographed some synthetic sympathomimetic amines, e.g., carbasil and epinine, which, like adrenaline, are catechol derivatives. Zweig (13) showed that sympathomimetic catecholamines formed multiple spots when they were chromatographed on paper from solution in 0.1*N* HCl, using a phenol-hydrochloric acid solvent system. The amines must have been present as hydrochlorides under these conditions because the same acid was being used in the salt and the developing solvent. Zweig (13) confirmed that complexes had been formed by techniques other than paper chromatography. These are only a few of the many possible references that could be mentioned.

We have developed chromatograms of 5 sympathomimetic amines with different organic solvents with water as the stationary phase. The amines were satisfactorily separated in the 2-solvent systems examined.

METHOD

Reagents

(a) *Solvents.*—Nanograde or spectrograde benzene, *n*-hexane, ethyl acetate, *n*-butanol, toluene, *m*-xylene, ethanol, and *n*-propanol (Fisher Scientific Co., 1241 Ambassador Blvd, St. Louis, MO 63132); ACS grade glacial acetic acid and ammonium hydroxide.

(b) *p-Nitrobenzoyl chloride solution.*—**0.4%.** Dissolve **400** mg *p*-nitrobenzoyl chloride (Eastman Kodak Co., Rochester, NY **14650**, No. p-**499**) in **10** ml ethyl acetate and dilute to **100** ml with *n*-hexane.

(c) *Bromocresol green.*—**0.5%.** Dissolve **500** mg bromocresol green (Matheson, Coleman & Bell, Norwood, OH **45212**, No. NB **179**) in **100** ml

Table 1. R_f values on ITLC fiber sheet of some sympathomimetic amines in 2-solvent systems, after treatment with *p*-nitrobenzoyl chloride

Compound[a]	Benzene-ethyl acetate (70+30)		Benzene-ethyl acetate (30+70)		Hexane-ethyl acetate (50+50)		Hexane-ethyl acetate (30+70)[b]	
1	0.10	0.36	0.14	0.71	0.11	0.43	0.13	0.65
2	0.10	0.79	0.12	0.90	0.09	0.76	0.12	0.90
3	0.11	0.29	0.12	0.56	0.12	0.28	0.12	0.49
4	0.13	0.84	0.14	0.95	0.10	0.75	0.13	0.94
5	0.12	0.64	0.14	0.92	0.12	0.78	0.12	0.88
Reagent	0.12	—	0.12	—	0.10	—	0.12	—

[a] 1, ephedrine hydrochloride; 2, phenylpropanolamine hydrochloride; 3, *N*-phenylethanolamine; 4, hydroxyamphetamine hydrobromide; 5, propylhexedrine (all standards 10 mg/ml 0.1*N* HCl).
[b] 20 min developing time.

ethanol. Dilute 1 ml aliquot to 100 ml with ethanol.

(d) *Sympathomimetic amine standard solutions.*—(*1*) Weigh 1 g of each amine (phenylpropanolamine hydrochloride, ephedrine hydrochloride, *N*-phenylethanolamine, hydroxyamphetamine hydrobromide, and propylhexedrine) into separate 100 ml volumetric flasks and dilute to volume with 0.1*N* HCl (10 mg/ml). (*2*) Weigh 500 mg of each amine into separate 100 ml volumetric flasks. Add 10 ml water and dilute to 100 ml with ethanol (5 mg/ml).

Apparatus

(a) *TLC fiber sheet.*—Gelman ITLC fiber SAF (instant thin layer chromatogram, salicylic acid fluorescent media, Gelman Instrument Co., Ann Arbor, MI 48106). Fiber sheets can be used without activation.

(b) *Glass plates.*—Precoated glass plates for TLC (Analabs, Northhaven, CT 06473), Anasil G type, 20 × 20 cm, 500 μm thick.

(c) *Developing tanks.*—Stainless steel or glass, 9 × 9 × 4″.

(d) *Ultraviolet (UV) light.*—Black-Ray UVL-22 (Ultraviolet Products, Inc., San Gabriel, CA 91778), 115 v, 60 cycles, equipped with short and long wavelengths.

Procedure

(a) *For ITLC fiber sheet.*—Apply 5 μl standard solutions 1 side by side on fiber chromatogram sheet 1.5 cm from bottom, and let spots dry. Over each spot, apply 5 μl *p*-nitrobenzoyl chloride reagent, and let spots air-dry. Develop chromatogram sheet to line 10–12 cm above spot (ca 20 min). Remove chromatogram sheet and dry in 100°C oven ca 1 min. Examine derivative spots (yellow) under shortwave UV light. Determine R_f value for each spot.

(b) *For glass plate.*—Apply 5 μl standard solutions 2 to glass plate 25 cm from bottom and 40 mm apart (do *not* touch plate with pipet). Let spots dry. Over each spot apply 5 μl bromocresol green, and let spots air dry. Amines are indicated by blue spots on greenish yellow background; spots will turn yellow in acetic acid vapors. Develop each chromatogram to line 15–20 cm above origin spot (ca 45 min). Remove plate from tank and dry in 100°C oven ca 3 min. Examine derivative spots (green) under shortwave UV light. Determine R_f value for each spot.

Results

A solution of sympathomimetic amine in 0.1*N* HCl showed 2 spots when treated with *p*-nitrobenzoyl chloride and chromatographed in the benzene-ethyl acetate and hexane-ethyl acetate solvent systems. The reagent itself gives one spot in this system. Table 1 indicates the R_f values of standards in the 2-solvent systems. The more polar spots all have R_f values which are approximately the same, and the less polar spots have different R_f values, thus producing separation.

A solution of amine in ethanol showed 1 spot when treated with bromocresol green and chromatographed in the ethanol-water-ammonium hydroxide, *n*-propanol-water-acetic acid, and *n*-butanol-water-acetic acid solvent systems. In addition, the reagent gives 1 spot. By using these systems, we cannot identify any amines.

A solution of amine in ethanol showed 1 and 2 spots when treated with bromocresol green and chromatographed in the *n*-butanol-toluene-water-acetic acid and *n*-butanol-*m*-xylene-water-acetic acid solvent systems, while the reagent gives one spot. Table 2 indicates the R_f values of standards in the 4-solvent systems. Only propylhexedrine is satisfactorily separated from the other amines.

Received August 13, 1975.

Table 2. R_f values on Anasil G plate of some sympathomimetic amines in 4-solvent systems, after treatment with bromocresol green

Compound[a]	*n*-Butanol-toluene-water-acetic acid (10+90+10+40)	*n*-Butanol-toluene-water-acetic acid (15+85+10+40)	*n*-Butanol-toluene-water-acetic acid (20+80+10+40)[b]	
1	0.08	0.12	0.18	0.54
2	0.09	0.14	0.20	0.56
3	0.05	0.11	0.16	—
4	0.06	0.11	0.18	—
5	0.16	0.24	0.31	—
Reagent	0.05	0.10	0.16	—

[a] See Table 1 (all standards 5 mg/ml ethanol.
[b] 45 min developing time.

By using several 2-solvent systems, we can identify some amines. In the 3- and 4-solvent systems, we can identify only one amine in the presence of others. We could not identify amines in pharmaceuticals because only one spot was formed. Continued study is recommended.

Acknowledgments

The authors wish to thank the Woodson-Tenent Laboratories for their cooperation and research facilities and Raymond C. Grant, National Center for Drug Analysis, Food and Drug Administration, St. Louis, MO, for helpful discussion about the project.

REFERENCES

(1) West, G. B. (1959) *J. Pharm. Pharmacol.* **11**, 595–599
(2) Beckett, A. H., Beaven, M. A., & Robinson, A. E. (1960) *Nature London* **186**, 775–776
(3) Beckett, A. H., Beaven, M. A., & Robinson, A. E. (1961) *J. Pharm. Pharmacol. Suppl.* **12**, 203T–216T
(4) Robinson, B., & Shepherd, D. M. (1961) *J. Pharm. Pharmacol.* **13**, 374–377
(5) Roberts, D. J. (1964) *J. Pharm. Pharmacol.* **16**, 549–556
(6) Roberts, D. J., & Broadly, K. J. (1966) *J. Pharm. Pharmacol.* **18**, 182–187
(7) Beckett, A. H., & Choulis, N. H. (1963) *J. Pharm. Pharmacol. Suppl.* **15**, 236T–245T
(8) Beckett, A. H., & Choulis, N. H. (1963) XXIII Internationaler Kongress der pharmazeutischen Wissenschafter, Munster, Germany
(9) Choulis, N. H. (1964) Ph.D. Thesis, London University, London, England
(10) James, W. O. (1948) *Nature London* **161**, 851–852
(11) James, W. O., & Kilbey, N. (1951) *J. Pharm. Pharmacol.* **3**, 22–26
(12) Munier, R., & Macheboeuf, M. (1949) *Bull. Soc. Chem. Biol. Paris* **31**, 1144–1162
(13) Zwieg, G. (1959) *Anal. Chem.* **31**, 821–824

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee B and was accepted by the Association. *See* (1976) *JAOAC* **59**, 383.

This report of the Associate Referee, J. J. Shah, was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

TECHNICAL COMMUNICATION

Confirmation of Results of Rapid Screening Test for Aflatoxins Performed at Corn Elevator

ODETTE L. SHOTWELL, GAIL M. SHANNON, and MARION L. GOULDEN
Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604

A screening method for corn and corn products, based on a minicolumn, was modified slightly to assay 60 lots of corn at one elevator to determine whether they could be sold as animal feed. To be salable, the lots had to contain less than 20 ppb total aflatoxin. Aflatoxin levels in the lots were later determined by the official AOAC method for corn to check effectiveness of the screening. No lot had been designated for sale that contained 20 or more ppb total aflatoxin.

In 1974–1975, there was a need to devise a reliable testing program that could be carried out at an elevator. In 1972, white corn under loan from the 1971 crop year had been delivered to an elevator in southeastern Missouri and placed in 3 storage bins (1), depending on rapid screening tests for aflatoxin and corn quality. The bins were supposed to be for aflatoxin-free, aflatoxin-contaminated, and U.S. Sample Grade (SG) corn. Samples that contained the bright greenish yellow (BGY) fluorescence associated with the presence of aflatoxin (2, 3) were subjected to a rapid minicolumn test (4). This test was done on a 50 g sample selected from a 5 lb probe sample to contain as much BGY material as possible. Probably because of sample selection, a great deal of aflatoxin-free corn was placed in the aflatoxin-contaminated bin, as determined by the CB method later performed at the Northern Laboratory on 10 lb samples that were ground and blended.

The Food and Drug Administration agreed that lots of corn from the contaminated and SG bins could be sold as animal feed if they contained <20 μg total aflatoxin/kg. As lots were unloaded at the elevator, they were analyzed according to the minicolumn method devised by Barabolak *et al.* (5) with minor changes that decreased the sensitivity. These minicolumn results were checked by analyzing the same samples later at the Northern Laboratory by the CB method.

Presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Experimental

Sampling at Elevator

A 50 lb continuous sample from every 2200–2500 bushel lot of corn was taken with a primary sampler (Dean Gomet Series 6800, vane-type) during unloading. From each 50 lb of corn, a rotary divider (Dean Gomet Series 4000) split out a 10 lb subsample to be ground in a 6″ Raymond hammermill containing ⅛″ roundhole perforations to pass a No. 20 sieve. Ground samples were blended 15–30 min in a Hobart Model A200 planetary mixer (12 qt capacity).

Rapid Screening Method (Minicolumn)

(a) *Preparation of column.*—Plug tapered end of glass tube (6 mm id × 23 cm long, tapered at one end) with 5 mm glass wool. Add in order: 8–10 mm Drierite, 8–10 mm Florisil, 16–20 mm silica gel, 8–10 mm neutral alumina, 8–10 mm Drierite (5). Plug top of column with glass wool. Tap after each addition to form firm layers. Apply slight pressure to glass wool plug at top to make certain column is tightly packed.

(b) *Preparation of extract.*—Follow the method of Barabolak *et al.* (5) with the following exceptions: Transfer 5 ml filtrate from ammonium sulfate precipitation to 125 ml separatory funnel. Redissolve residue from benzene extraction in 6.0 ml $CHCl_3$-acetone (9+1).

Prepare blank in identical manner from aflatoxin-free corn. Prepare standard similarly, but to residue from benzene extraction add 13 μl acetonitrile-benzene (2+98) solution containing 1.0 μg B_1 and 0.3 μg B_2/ml, and then redissolve in 6.0 ml $CHCl_3$-acetone (9+1).

(c) *Chromatographic analysis.* — With pipet, transfer 2 ml each of blank sample, standard extract, and unknown extracts onto series of columns; let columns drain completely. To each column add 1.0 ml $CHCl_3$-acetone (9+1) and again let drain. Observe fluorescence under ultraviolet light (365 nm). Presence of aflatoxin in corn sample results in blue fluorescent band at top of Florisil layer that can be identified by comparison with standard. Sample fluorescence with same or greater intensity than that in standard

Table 1. Distribution of aflatoxin levels in corn samples determined to contain <20 or ≥20 μg/kg by minicolumn test at elevator

<20 μg/kg by minicolumn		≥20 μg/kg by minicolumn	
Total aflatoxin, CB method, μg/kg	No. of samples	Total aflatoxin, CB method, μg/kg	No. of samples
0–<7	0	0–<13	0
7–10	10	13–19	9
11–15	7	20–29	8
16–18	3	30–39	9
		40–49	3
		50–59	6
		60–69	2
		70–79	2
		80–89	1

column indicates total aflatoxin content ≥20 μg/kg. If total aflatoxin content of corn sample is ≤10 μg/kg, a blue fluorescent band should not appear on the minicolumn with the modifications in method as described. When extracts of corn containing 10 μg total aflatoxin/kg are chromatographed, there will be no more than a trace of blue fluorescence.

CB Method for Corn

Proceed as in 26.014–26.019 (6).

Results and Discussion

Changes were made in the screening method described by Barabolak *et al.* (5) to decrease sensitivity, e.g., to increase the minimum concentration that could be detected. At the elevator in southeastern Missouri, the only interest was in knowing whether a given lot of corn contained <20 μg/kg and could be sold for feed. The detection limit of the method as published is 0.5 μg total aflatoxin/kg if a 4 mm id glass tube is used for minicolumn chromatography and 1 μg/kg if the glass tube is 6 mm id. We chose the 6 mm id glass tubes because they are available commercially. We also decreased the volume of the filtrate from the ammonium sulfate precipitation from 100 to 5 ml. Thus an extract from corn containing 20 μg aflatoxin/kg would give a clearly defined blue fluorescent band on the minicolumn under ultraviolet light (365 nm), but an extract from corn containing 10 μg toxin/kg would not. A technician, therefore, could decide more easily whether the original corn contained <20 μg aflatoxin/kg. By either decreasing or diluting the volume of the filtrate, or both, one can set the detection limit of the method at any level.

Fifty-eight lots of corn containing 2200–2500 bushels were unloaded at the elevator. Two lots

Table 2. Aflatoxin in corn as delivered to elevator in 1972 and as unloaded in 1974–1975

	Approx. distribution of aflatoxin in corn delivered to contaminated[b] and SG[c] bins		Aflatoxin distribution in corn unloaded from contaminated and SG bins	
Aflatoxin,[a] μg/kg	No. of lots	% of total	No. of lots	% of total
ND[d]	203	41	0	0
<10	79	16	8	13
10–19	71	14	21	35
20–29	35	7	8	13
30–39	18	4	9	15
40–49	13	2	3	5
50–59	13	2	6	10
60–69	15	3	2	3
70–79	5	1	2	3
80–89	9	2	1	2
90–99	11	2	0	0
>100	29	6	0	0
Total	501	100	60	99

[a] As determined by CB method 26.014–26.019 (6).
[b] Delivery to contaminated bin depended on results of minicolumn test.
[c] SG = U.S. Sample Grade.
[d] ND = Not detected.

contained 1400–1600 bushels. Out of the 60 lots, 20 contained <20 μg aflatoxin/kg on the basis of the modified minicolumn test on 10 lb blended, ground samples. These lots were sold for animal feed in the vicinity of the elevator. When the same 10 lb samples were analyzed by the CB method at the Northern Laboratory, none contained 20 μg aflatoxin/kg (Table 1). In fact, most lots sold contained <15 μg toxin/kg. Quantitative analysis at the Northern Laboratory of the 40 corn samples that gave positive minicolumn tests (≥20 μg aflatoxin/kg) at the elevator revealed 31 contained 20 μg aflatoxin/kg (Table 1). Nine samples contained between 13 and 19 μg/kg. The minicolumn screening method proved to be effective in determining which lots of corn contained <20 μg aflatoxin/kg.

When the corn was placed in the so-called aflatoxin-contaminated and SG bins in southeastern Missouri in 1972 (1), the average level of aflatoxin was 27 μg/kg, assuming each of the 501 truckloads (200–400 bushels) had the same capacity. The average level of toxin as unloaded in sixty 1400–2500 bushel lots was 28 μg/kg. There was no appreciable increase in toxin level during the 2 years of storage. There appeared to be blending of the lots as the corn was placed in the bins and taken out (Table 2). Aflatoxin was not detected in 41% of the truckloads placed in the bins. When the corn was unloaded in lots of 1400–2500 bushels, no lot was found to be aflatoxin-

free (sensitivity of assay is 1–3 μg/kg). Only one lot had more than 80 μg toxin/kg.

Acknowledgment

We thank the Agricultural Stabilization and Conservation Service, U.S. Department of Agriculture, for providing the corn samples from southeastern Missouri and also the results of the screening tests.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Received April 15, 1976.

REFERENCES

(1) Shotwell, O. L., Shannon, G. M., & Hesseltine, C. W. (1975) *Cereal Chem.* **52**, 381–387

(2) Shotwell, O. L., Goulden, M. L., & Hesseltine, C. W. (1972) *Cereal Chem.* **49**, 458–465

(3) Shotwell, O. L., Goulden, M. L., & Hesseltine, C. W. (1974) *Cereal Chem.* **51**, 492–499

(4) Shannon, G. M., Stubblefield, R. D., & Shotwell, O. L. (1973) *JAOAC* **56**, 1024–1025

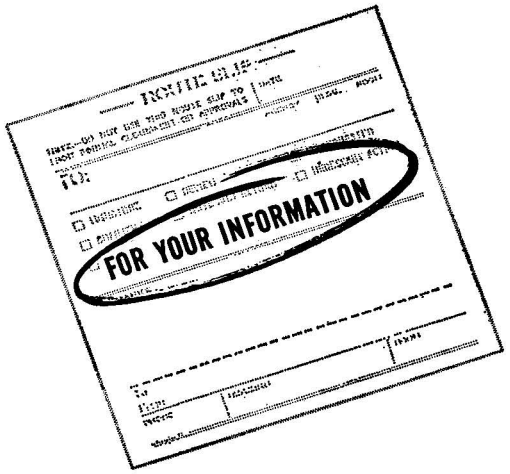
(5) Barabolak, R., Colburn, C. R., & Smith, R. J. (1974) *JAOAC* **57**, 764–766

(6) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC

CORRECTION

JAOAC **59**, 830–834 (1976), "Colorimetric Determination of Zirconium in Antiperspirant Aerosols," by Paul Beavin, Jr., p. 832, left column, line 15

Change to read "of clean, dry can (without cap) and dip tube from gross"



XX International Dairy Congress to be Held in Paris in 1978

The XX International Dairy Congress will be held at the Centre International de Paris in Paris, France, June 26–30, 1978. Mr. Jean Raffarin, former Minister of Agriculture and President of the French National IDF Committee, serves as President of Congrilait 78.

The scientific and technical program, headed by Professor Andre Francois, will include discussions and lectures on the nutritional value of milk; factors of acceptability of dairy products; food habits and consumption of dairy products; new technological processes, particularly in energy and water conservation and environmental protection; milk production; bacteriology and biochemistry of milk and dairy products; and new products.

The economic and commercial program, headed by M. Jean Mittaine, member of the IDF Executive Committee, will consider how to develop milk production in different types of agriculture based on breeding cows, buffalos, goats, or ewes; and how to harmonize relations among milk production, manufacturing, and consumption.

Reports will be published in a volume to be distributed at the beginning of Congrilait 78. Official languages will be French, German, and English. Simultaneous translations will be offered. During the Congress, technical visits to dairies, dairy farms, goat and sheep farms, and research laboratories will be organized. Also on the agenda are excursions to different regions of France before and after the meeting. For further information, write: Congrilait, 50 rue Fabert, F-75007 Paris, France.

New: Journal of Chromatography, Biomedical Applications

Elsevier Scientific Publishing Company will publish starting January 1977 the *Journal of Chromatography, Biomedical Applications.* Issued bimonthly, the journal will be devoted to new developments and advances in biomedical applications of chromatography and electrophoresis. It will form an integral part of the *Journal of Chromatography,* but can also be subscribed to separately. The subscription price for 1 year is $49 U.S./Dfl 124.00, including postage.

The journal will publish papers dealing with developments and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including publishing of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combination of chromatographic and electrophoretic methods with other physicochemical techniques like mass spectrometry. Original papers, short communications, review articles, information on new instrumentation and forthcoming events, and book reviews will be considered for publication.

Free sample copies will be available on request from: Elsevier Scientific Publishing Co., PO Box 211, Amsterdam, The Netherlands.

NBS Conference Briefs

The National Bureau of Standards of the U.S. Department of Commerce will sponsor the following meetings in the coming months:

November 8–12—Computer Performance Evaluation Users Group, San Diego, CA. Sponsored by NBS and Computer Performance Evaluation Users Group. Contact: Dick Dunlavey, A265 Technology Building, NBS, Washington, DC 20234. 301/921-3485.

November 17—Computer Networking Symposium, NBS, Gaithersburg, MD. Sponsored by NBS and the Institute of Electrical and Electronics Engineers' Technical Committee on Computer Communications. Contact: Marshall Abrams, B212 Technology Building, NBS, Washington, DC 20234. 301/921-2601.

November 29–December 1—Bicentennial Mathematical Programming Symposium, NBS, Gaithersburg, MD. Sponsored by NBS and the Special Interest Group on Mathematical Programming of the Association for Computing Machinery. Contact: William Hall, A422 Administration Building, NBS, Washington, DC 20234. 301/921-3855.

December 6–8—Winter Simulation Conference, NBS, Gaithersburg, MD. Sponsored by NBS, Association for Computing Machinery, American

Institute of Industrial Engineers, Institute of Electrical and Electronic Engineers, Operations Research Society of America, Society for Computer Simulation, and the Institute of Management Sciences. Contact: Paul Roth, B250 Technology Building, NBS, Washington, DC 20234. 301/921-3545.

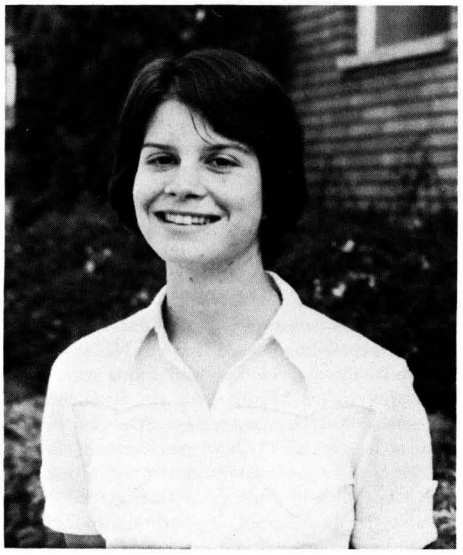
January 10–13, 1977—Phase Diagrams in Metallurgy and Ceramics, NBS, Gaithersburg, MD. Sponsored by NBS. Contact: Lawrence Bennett, B152, Materials Building, NBS, Washington, DC 20234. 301/921-2982.

Jana Ruth Ostrom Receives 1976 AOAC Scholarship

Jana Ruth Ostrom, an outstanding pharmacy student at the University of Washington, is the 1976 winner of a $1000 scholarship sponsored by the Association of Official Analytical Chemists.

Mrs. Ostrom, who resides in Seattle, WA, holds a B.S. in zoology (1974, cum laude) from Johns Hopkins University. She is working toward her second bachelor's degree at the University of Washington's School of Pharmacy and has maintained a 4.0 grade point average during her first 2 years of study. She has worked in science laboratories since 1972 and is currently a pharmacy intern with the U.S. Public Health Service in the State of Washington.

Each year AOAC awards the 2-year scholarship to a college sophomore who is studying a subject important to public health and agriculture. To qualify, the student must be in need of financial aid, maintain at least a B average during the first 2 years of undergraduate study, and plan to do research, regulatory work, or quality control, or teach in an area of interest to the AOAC. Past scholarship winners have studied at Oregon State, California State, Rutgers, Clemson, Purdue, Carleton College, St. Joseph's College, and the University of Nebraska.



Nominations for the 1977 award must be received before May 1, 1977. Six copies of a nomination letter and 2 supporting reference letters should be sent to: Executive Secretary, AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20044.

AOAC to Hold Second Annual Spring Meeting

The Association of Official Analytical Chemists (AOAC) will hold its second Annual Spring Workshop May 4–6, 1977, at the Stouffer's Inn in Cincinnati, OH.

Eight work sessions are planned for the 3-day conference. The topics discussed will include:

(*1*) *Vitamins.*—High-pressure liquid chromatography (HPLC) and fluorescent techniques for vitamins A, B_1, B_2, C, D, and E.

(*2*) *Mycotoxins.*—Thin layer chromatographic and HPLC methodology for detection of toxins related to animal and human health.

(*3*) *Computer adaptation to laboratory instruments.*—Mini-computers with on-line capabilities or potential.

(*4*) *Food microbiology.*—Methods for detecting bacteria, molds, or viruses in various commodities, meats, water, and canned food. Laboratory standardization of techniques and automated techniques as well as the fluorescent antibody procedure will be discussed.

(*5*) *Medicated feeds.*—Colorimetric and polarographic methods for drug additives as well as detecting of antibiotics by automated, turbidimetric, and plate assays.

(*6*) *Environmental contaminants.*—Determining heavy metals, pesticides, and other industrial chemicals by atomic absorption spectroscopy, HPLC, gas-liquid chromatography, and polarography. Methodology for field testing will also be covered.

(*7*) *Insect identification.*—A 2½ day workshop will be conducted by Food and Drug Administration (FDA) experts.

(*8*) *Staphylococcal enterotoxin.*—A 2½ day workshop will be conducted by FDA experts at the district office for the first 20 registrants.

Registration will be held Wednesday morning, May 4, 1977. The registration fee is $15. Reservations may be made at Stouffer's by calling directly (513/721-8600). Rates are $25 for a single and $33 for a double room.

For further information contact: Howard P. Moore, Chief, Consumer Analytical Laboratories, Ohio Department of Agriculture, Reynoldsburg, OH 43068 (614/866-6361); or John Feldman, Laboratory Director, Food and Drug Administration, 1141 Central Parkway, Cincinnati, OH 45202 (513/684-3511).

W. W. Wright, New AOAC President

William Wynn Wright, Ph.D., was installed as the new president of the AOAC at the Association's 90th Annual Meeting in Washington, DC.

Dr. Wright is Deputy Associate Director, Pharmaceutical Research and Testing, of the Bureau of Drugs, U.S. Food and Drug Administration (FDA). He has served the AOAC from 1957 to 1971 as General Referee on Antibiotics, as Executive Committee member since 1972, and as AOAC Vice-President in 1975.

Born in Baltimore, MD in 1923, Dr. Wright received his B.S. degree in chemistry and mathematics (magna cum laude) from Loyola College in 1944, and his M.S. and Ph.D. degrees in 1946 and 1948, respectively, from Georgetown University.

He began his career as a chemist with the National Bureau of Standards in 1945; then transferred to FDA's newly formed antibiotic certification program. Dr. Wright is now responsible for the Pharmaceutical Research and Testing activities of the Bureau of Drugs, providing technical guidance and participation in scientific research programs to develop and improve biological,

microbiological, chemical and instrumental methods of drug analysis and to establish the pharmacological and toxicological properties of drugs.

Since 1960, he has also served as a consultant on antibiotics and biological standardization for the World Health Organization. He received a superior Service Award in 1964 from the Department of Health, Education and Welfare, and an Award of Merit in 1971 from the FDA; he was an AOAC Fellow in 1969. Dr. Wright is a member of the American Association for the Advancement of Science, New York Academy of Sciences, American Pharmaceutical Association, Academy of Pharmaceutical Sciences, and the Research Society of America.

BOOK REVIEWS

Electroanalytical Chemistry. H. W. Nurnberg (Ed.). John Wiley & Sons, Inc., New York, NY, 1974. xi + 609 pp. Price $49.50.

This book is Vol. 10 of the Advances in Analytical Chemistry and Instrumentation Series. It consists of 7 chapters by various international experts who discuss selected topics in electroanalytical chemistry. All of the chapters are well documented, containing a total of 1776 references.

Chapter 1 by D. J. Fisher is a lengthy and informative discussion on the design, testing, and uses of instrumentation for dc polarography and for coulometry, geared to the needs and requirements of the electroanalytical chemist. The 4 principal subject areas are: system concepts as applied to polarography, measurement systems for dc polarography, instrumentation for coulometry, and digital systems for automated electroanalytical chemistry. Using polarographs and coulometers developed at Oak Ridge National Laboratory as models, the author describes in great detail their development and component parts. Various performance tests used in the development of these instruments can readily serve to check performance characteristics of similar electrochemical instruments.

In Chapter 2, G. W. C. Milner and G. Phillips present an excellent review of the applications of voltammetry and coulometry in the analysis of the actinide elements. The review is somewhat dated in that its most recent references were published in 1968. However, this chapter should be helpful to anyone engaged in the analysis for these important elements.

P. J. Elving presents in Chapter 3 a timely and interesting discussion of the current and future trends in the use of voltammetric techniques in organic analysis and in the study of organic reaction kinetics. In order to portray as realistically as possible the extent to which polarographic and voltammetric techniques are actually being used in organic analysis, especially from the service aspect, the author canvassed over 30 laboratories representing industry, government, and educational institutions. Many of the specific comments received from these laboratories are incorporated in the present review and should be of valuable assistance for anyone contemplating using these powerful electroanalytical techniques for organic analysis and for organic reaction kinetic studies.

H. Hoffman and J. Volke present in Chapter 4 a very comprehensive review on the development and applications of the various voltammetric techniques to production control and to the qualitative and quantitative analysis of drugs in unknown preparations and to the identification and quantitation of pharmaceuticals in fluids of the human and/or animal body with respect to toxicology, pharmacology, and pharmacological kinetics. There are many practical suggestions for using electroanalytical techniques to analyze formulations, tablets, ointments, creams, and similar pharmaceutical preparations for major drug ingredients and trace contaminants. Future trends and possibilities for using polarography and voltammetry in the pharmaceutical industry and pharmacological studies are discussed.

In Chapter 5, J. Badoz-Lambling and G. Cauquis survey the basic concepts involved in studies of electrochemical phenomena occurring in nonaqueous solutions and various fused salt melts. Many examples are from their own experiences in nonaqueous electrochemistry with emphasis on the practical aspects of the subject. Included are sections on *in situ* visible and ultraviolet electrochemical spectroscopy and the separation and identification of reaction products and trace contaminants.

B. Kastening presents in Chapter 6 an excellent review of the joint applications of electrochemical and ESR techniques for detecting and identifying radicals involved in electrochemical reactions, thus elucidating unknown mechanisms or confirming those derived from electrochemical and/or other techniques of stimulating organic reactions in solutions. A vast number of topics such as experimentation, theory, mechanisms, and conformation has been very well organized into a short, understandable, and readable chapter.

K. Schwabe in the final chapter presents an excellent discussion of the theory and newer applications of pH measurements to industrial process control and to the biological-medical field. He discusses the requirements for accuracy and reproducibility which have been raised by these newer applications and the problems encountered in attempting to meet them. This timely chapter should interest anyone involved in pH measurements of any kind.

The book more than adequately fulfills the requirement of the Advances Series in that it contains critical comprehensive articles surveying various topics on a high level which are satisfying to the specialist and nonspecialist alike.

RAYMOND J. GAJAN
Food and Drug Administration
Washington, DC

A Dictionary of Chromatography. R. C. Denney. Halsted Press, John Wiley & Sons, Inc., New York, NY, 1976. xi + 191 pp. Price $14.50.

This book provides concise, easily understandable definitions in alphabetical order of abbreviations, terms, and expressions frequently employed in all fields of chromatography from adsorption to zone electrophoresis. One of the author's objects in writing the dictionary is to save readers the effort of wading through several advanced chromatography books in order to obtain answers to relatively straightforward questions. Dr. Denney has also provided over 300 references to both journals and textbooks, complete through 1975, so that more detailed studies can be pursued where required.

Despite efforts to standardize chromatographic terms and nomenclature, there is still much confusion in this area. Where possible the author has used those terms which appear to be generally accepted, and at the same time to indicate how these are interrelated throughout the various forms of chromatography. Because detectors are identified by a variety of names in the chemical literature, Dr. Denney has included as many of these as possible, cross-referencing them in every case to the name that seems to be most frequently employed.

A Dictionary of Chromatography is not intended for experts in chromatographic theory and practice. It provides an excellent reference source for the student, laboratory technician, or general scientist seeking rapid information on chromatography. Besides the principal equations covering theoretical chromatography, the book describes the equipment available for carrying out chromatography in all its forms, with the aid of over 50 diagrams and illustrations. The price appears somewhat high, but it turns out to be a good value for the quality and quantity of information provided.

ALAN J. SENZEL

AOAC
Washington, DC

NEW PUBLICATIONS

Approved Methods of the American Association of Cereal Chemists. American Association of Cereal Chemists, 334 Pilot Knob Rd, St. Paul, MN 55121, 1976. 950 pp. Price $75 to members; $100 to nonmembers.

This 2-volume loose-leaf set containing 352 methods furnishes an updated version of AACC methods for cereal and related products.

Treatise on Analytical Chemistry. Part I: Theory and Practice. Vol. 11. Edited by U. M. Kolthoff and Philip J. Elving with assistance of Ernest B. Sandell. John Wiley & Sons, Inc., New York, NY, 1975. xxiii + 697 pp. Price $39.50.

This volume treats in great detail general laboratory apparatus, general laboratory operations and techniques, safety, common apparatus, techniques, and operations, including safety, and classical methods of analysis (quantitative analysis, gravimetric and titrimetric analysis). Many of the examples are from inorganic analysis, metallurgy and rock analysis, where the term residue has its original analytical meaning of that which remains after an operation, rather than the extremely small amounts of materials sought in environmental analysis. Much of the general first section is so basic that it reads much like a text of 40 years ago. It is this type of fundamental information which makes this volume valuable.

International Standards for Agricultural Equipment and Supplies. ISO Information Centre Bibliography No. 4, June 1976. International Organization for Standardization, Case Postale 56, 1211 Geneva 20, Switzerland. 5 pp. plus annexes. Price Sw 9 ($3.60).

This bibliography contains the ISO International Standards, draft International Standards, and draft proposals for the following products: machinery, propagation materials, pesticides, and fertilizers. Particularly valuable is the list of other reference sources of international information and names and addresses of international organizations active in these fields.

AUTHOR INDEX

U.S. POSTAL SERVICE

STATEMENT OF OWNERSHIP, MANAGEMENT AND CIRCULATION

(Required by 39 U.S.C. 3685)

1. TITLE OF PUBLICATION: JOURNAL OF THE ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS		2. DATE OF FILING: OCT. 29, 1976
3. FREQUENCY OF ISSUE: BI-MONTHLY	A. NO. OF ISSUES PUBLISHED ANNUALLY: SIX	B. ANNUAL SUBSCRIPTION PRICE: $36.00

4. LOCATION OF KNOWN OFFICE OF PUBLICATION *(Street, City, County, State and ZIP Code) (Not printers)*: 200 C STREET, S. W., WASHINGTON, D. C. 20204

5. LOCATION OF THE HEADQUARTERS OR GENERAL BUSINESS OFFICES OF THE PUBLISHERS *(Not printers)*: 200 C STREET, S. W., WASHINGTON, D. C. 20204

6. NAMES AND COMPLETE ADDRESSES OF PUBLISHER, EDITOR, AND MANAGING EDITOR

PUBLISHER *(Name and Address)*: ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, BOX 540, WASHINGTON, D. C. 20044

EDITOR *(Name and Address)*: MISS HELEN REYNOLDS, BOX 540, WASHINGTON, D. C. 20044

MANAGING EDITOR *(Name and Address)*: MRS. NANCY PALMER, BOX 540, WASHINGTON, D. C. 20044

7. OWNER *(If owned by a corporation, its name and address must be stated and also immediately thereunder the names and addresses of stockholders owning or holding 1 percent or more of total amount of stock. If not owned by a corporation, the names and addresses of the individual owners must be given. If owned by a partnership or other unincorporated firm, its name and address, as well as that of each individual must be given.)*

NAME	ADDRESS
ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS	BOX 540, WASHINGTON, D. C. 20044
NON-PROFIT ORGANIZATION	
NO STOCKHOLDERS	

8. KNOWN BONDHOLDERS, MORTGAGEES, AND OTHER SECURITY HOLDERS OWNING OR HOLDING 1 PERCENT OR MORE OF TOTAL AMOUNT OF BONDS, MORTGAGES OR OTHER SECURITIES *(If there are none, so state)*

NAME	ADDRESS
NONE	

9. FOR COMPLETION BY NONPROFIT ORGANIZATIONS AUTHORIZED TO MAIL AT SPECIAL RATES *(Section 132.122, PSM)*

The purpose, function, and nonprofit status of this organization and the exempt status for Federal income tax purposes *(Check one)*

☐ HAVE NOT CHANGED DURING PRECEDING 12 MONTHS ☐ HAVE CHANGED DURING PRECEDING 12 MONTHS *(If changed, publisher must submit explanation of change with this statement.)*

10. EXTENT AND NATURE OF CIRCULATION	AVERAGE NO. COPIES EACH ISSUE DURING PRECEDING 12 MONTHS	ACTUAL NO. COPIES OF SINGLE ISSUE PUBLISHED NEAREST TO FILING DATE
A. TOTAL NO. COPIES PRINTED *(Net Press Run)*	5266	4975
B. PAID CIRCULATION 1. SALES THROUGH DEALERS AND CARRIERS, STREET VENDORS AND COUNTER SALES	NONE	NONE
2. MAIL SUBSCRIPTIONS	4506	4506
C. TOTAL PAID CIRCULATION *(Sum of 10B1 and 10B2)*	4506	4506
D. FREE DISTRIBUTION BY MAIL, CARRIER OR OTHER MEANS SAMPLES, COMPLIMENTARY, AND OTHER FREE COPIES	69	69
E. TOTAL DISTRIBUTION *(Sum of C and D)*	4575	4627
F. COPIES NOT DISTRIBUTED 1. OFFICE USE, LEFT OVER, UNACCOUNTED, SPOILED AFTER PRINTING	691	348
2. RETURNS FROM NEWS AGENTS	NONE	NONE
G. TOTAL *(Sum of E, F1 and 2—should equal net press run shown in A)*	5266	4975

11. I certify that the statements made by me above are correct and complete. SIGNATURE AND TITLE OF EDITOR, PUBLISHER, BUSINESS MANAGER, OR OWNER: Business Manager

12. FOR COMPLETION BY PUBLISHERS MAILING AT THE REGULAR RATES *(Section 132.121, Postal Service Manual)*

39 U. S. C. 3626 provides in pertinent part: "No person who would have been entitled to mail matter under former section 4359 of this title shall mail such matter at the rates provided under this subsection unless he files annually with the Postal Service a written request for permission to mail matter at such rates."

In accordance with the provisions of this statute, I hereby request permission to mail the publication named in item 1 at the phased postage rates presently authorized by 39 U. S. C. 3626.

SIGNATURE AND TITLE OF EDITOR, PUBLISHER, BUSINESS MANAGER, OR OWNER: Business Manager

PS Form 3526 Apr. 1976 *(Page 1)* (See instructions on reverse)

SUBJECT INDEX

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*) cautionary notes and comments on techniques, apparatus, and reagents; (*g*) reviews of methodology in special fields.

Preparation of Manuscript

Authors are required to submit three copies (one of which must be the original—ribbon—copy) of the complete manuscript, including all tables and all illustrations. The manuscript is to be typewritten on one side only of white bond paper, $8 \times 10\frac{1}{2}$ or $8\frac{1}{2} \times 11$ 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. Proper placement of tables and illustrations is taken care of by the editorial staff at the time the article is in proof.

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.

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. 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. Vertical and horizontal rules should be used sparingly; however, horizontal rules 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 drawings or photographs. 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 (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. *JAOAC* normally 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 *JAOAC*.

The compendium of methods of the Association should be listed as follows: *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, 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)
amp	ampere(s)
μa	microampere(s)
v	volt(s)
dc	direct current
′	foot (feet)
″	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
hr	hour(s)
min	minute(s)
sec	second(s)
%	per cent
$\overline{\$}$	standard taper
N	normal
M	molar
m*M*	millimolar

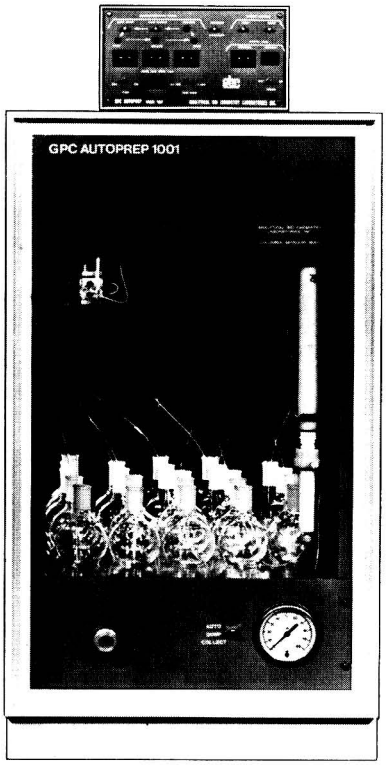
(Note: Spectrophotometric nomenclature should follow the rules contained in *Official Methods of Analysis*, "Definitions of Terms and Explanatory Notes.")

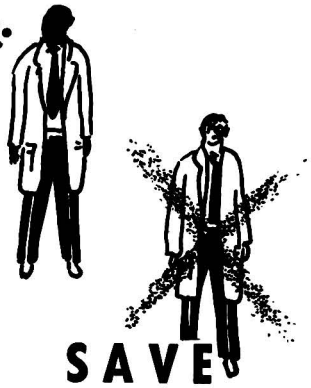
Rev. 5/76

AUTOMATE PESTICIDE RESIDUE CLEANUP.

SAVE 1/2 of SOLVENT and LABOR COSTS.

SAVE $1980





SAVE $7000+

23 SAMPLES AUTOMATICALLY PROCESSED

QUANTITATIVE RECOVERY OF PESTICIDES AND PCBS

ELIMINATES TIME CONSUMING MANUAL PARTITIONING

3.2% BETTER RECOVERY

The GPC 1001 Pays for itself in LESS THAN 1 YEAR Operated at One-Half Capacity While Providing Superior Results.

– UNTIL YOU GET ONE – LET OUR ANALYTICAL SERVICES QUOTE YOUR RESIDUE WORK

abc

ANALYTICAL BIOCHEMISTRY LABORATORIES, INC.
7200 EAST ABC LANE — P. O. BOX 1097
COLUMBIA, MISSOURI 65201
PHONE (314) 474-8579